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Mechanism of demethylation of Histone H3 Lysine 4 on *FLC* locus during vernalization depending on two JmjC-domain containing demethylases

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Dedication

To my family

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Mechanism of demethylation of Histone H3 Lysine 4 on *FLC* locus during vernalization depending on two JmjC-domain containing demethylases

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Flowering in plants is one of the crucial developmental events controlled by the multiple environmental stimuli. Vernalization is the response to the exposure to the prolonged cold to achieve the molecular competence for a subsequent floral transition. The main regulator of the vernalization response, *FLOWERING LOCUS C (FLC)*, is highly expressed to prevent precocious flowering before vernalization but becomes repressed during vernalization. The studies on the repression of *FLC* by vernalization have focused mostly on the transcriptional suppression of the locus achieved by components of POLYCOMB REPRESSION COMPLEX (PRC) and VERNALIZATION INSENSITIVE 3 (VIN3). These regulators contribute to the deposition of the repressive histone marks, the methylation of histone H3 lysine 27, at the *FLC* locus. However, the mechanisms to regulate other histone modifications during vernalization remain unclear. One of active histone marks, tri-methylated histone H3 lysine 4, is enriched at *FLC* before vernalization but the enrichment decreases by vernalization. Two JmjC-domain containing demethylases, JMJ16 and JMJ19, are induced by vernalization and are involved in the reduction of tri-methylated H3K4 at the *FLC* locus redundantly. These

demethylases, JMJ16 and JMJ19, are directly targeted to the *FLC* locus and interact with the cold-induced VIN3 protein. Another histone active mark, histone acetylation, is also highly enriched at the *FLC* locus. However, the mechanisms and regulators responsible for balancing the level of the acetylation at the *FLC* locus have not been fully characterized. The phenotypic analysis of the mutants of deacetylases revealed that two deacetylases, SRT1 and HDT1, are involved in the short day-specific flowering mechanism. The *srt1* and *hdt1* mutants exhibit the early flowering and the late flowering, respectively under short days in consistent with changes in expression of *FLC*. These results suggest that two histone deacetylases, SRT1 and HDT1, are associated with the short-day specific flowering regulation in *Arabidopsis* and may use the different regulatory mechanism on the target gene, *FLC*.

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Chapter 1. Flowering Pathways and regulation of *FLC*

1.1 Flowering pathways in *Arabidopsis*

Flowering is the transition from vegetative growth to the reproductive development of plants, which plays a crucial role in plants' life cycle. Plants have internal mechanisms that respond to various endogenous and environmental signals to achieve flowering at the proper time during a year and therefore maximize their reproductive success (Mylne et al., 2004). Long-day flowering plant *Arabidopsis thaliana* has been studied as a model system to understand complex genetic networks of flowering (Srikanth and Schmid, 2011). In *Arabidopsis*, six major flowering mechanisms have been identified and studied: photoperiod, vernalization, autonomous, gibberellin (GA), ambient temperature, and age pathway (Bohlenius et al., 2006; Song et al., 2012; Kim et al., 2009; Davis, 2009; Wang, 2014). These pathways eventually regulate floral meristem identity genes in the shoot apical meristem (SAM), resulting in flower organ development (Corbesier et al., 2007) (Fig 1).

Many plants use the relative change in the length of day and night (photoperiod) throughout the year to adjust their timing of flowering according to the seasonal variations. In *Arabidopsis*, *FLOWERING LOCUS T (FT)* encodes a florigen, and that the expression of *FT* is induced in the leaves under long days and FT protein moves to the shoot apex to promote flowering (Huang et al., 2005). The high level of *FT* expression is mediated by the transcriptional activator CONSTANS (CO) that also helps other floral integrator genes, including *SUPPRESSOR OF OVEREXPRESSOR OF CO 1 (SOC1)* (Putterill et al., 1995; Lee and Lee, 2010). GIGANTEA (GI) is an upstream regulator of *CO* and is involved in the circadian clock mechanism (Fowler et al., 1999). In the

photoperiod pathway, the module of GI-CO-FT takes the main role in floral transition in *Arabidopsis*.

The autonomous pathway mediate flowering independently of the photoperiod pathway. Genes in the autonomous pathway have been identified as repressors of the floral repressor, *FLOWERING LOCUS C (FLC)*. The autonomous pathway genes regulate the expression of *FLC* through RNA binding/processing or chromatin remodeling. For example, FCA, FPA, and FLK contain an RNA binding domain and, FY is an RNA-processing factor. FLD and REF6 are chromatin regulators mainly involved in histone demethylation (Kim et al., 2009; Baurle et al., 2008, Noh et al., 2004).

In *Arabidopsis*, gibberellic acid (GA) plays roles in various developmental programs, including germination, organ development, phase transitions, and floral initiation. The initiation of flowering that is promoted by GAs has been well studied and GAs function through the activation of floral integrator genes including *SOC1*, *LEAFY (LFY)*, and *FT* (Davis et al., 2009; Mutasa-Gottgens and Hedden, 2009). Mutations in genes involved in GA biosynthesis and signaling pathways affect the flowering time in *Arabidopsis*.

Vernalization is a response to the prolonged exposure to cold which results in the promotion of flowering in spring in many plant species (Song et al., 2012). The promotion of flowering by the long-term cold is achieved by the transcriptional repression of *FLC*, which encodes an MADS-box protein (Michaels and Amasino, 1999). FLC is a major floral inhibitor and suppresses the expression of a series of downstream floral integrators required for the floral transition. During vernalization, the slow but stable repression of *FLC* allows the release of several floral integrators, rendering the plants to be ready for flowering when the warm temperature comes back.

1.2 Vernalization pathway

In many plant species, the prolonged exposure to the cold temperature of the winter season accelerates flowering, and vernalization is a necessary process for flowering in some species. Especially in many crop species, the requirement of vernalization prevents the precocious flowering during the first growing season and allows the plant to flower at the favorable growth condition in the spring after the extended period of winter cold. Some strains of *Arabidopsis* also require vernalization treatment to achieve the acceleration of flowering. Vernalization response varies in the duration of cold exposure depending on plant species (Bernier et al., 1981; Chouard, 1960; Lang, 1965).

The competence to flower given by vernalization is stable, so that the competence is maintained even after plants are brought back to warm temperature. The molecular changes throughout the subsequent mitotic division during vernalization are stable to prevent plants from removing the effect of vernalization when the cold stimulus disappears (Dennis and Peacock, 2007; Kim et al., 2009). However, the memory of vernalization does not persist from one generation to the next generation because the molecular changes that were obtained by vernalization are not maintained when cells undergo meiosis. In the successive generation of plants, vernalization is again required to establish the competence to flower (Michaels and Amasino, 2000).

In vernalization, plants undergo two distinct processes, the perception of cold, and the stable change in gene expression. Optimal vernalization treatment requires the low temperature at near but above freezing temperatures and sufficient periods of cold exposure. The optimal temperature and the length of cold exposure vary depending on plant species (Lang, 1965). Therefore, how to perceive and measure the duration of cold

is an important mechanism to obtain the advantage of vernalization in plants. After the sufficient length of the proper cold exposure to plants, a cascade of changes in gene expression occurs, achieving mitotically stable repression of floral repressors genes and chromatin states followed by activation of floral integrator genes (Bastow et al., 2004; Sung and Amasino, 2004).

1.3 Regulation of *FLC* during the course of vernalization

FLC is a floral repressor that confers a requirement of vernalization to promote flowering in the following spring (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* encodes an MADS-box protein that functions as a transcriptional repressor by direct binding to several floral promoting genes, including *FT* and *SOC1* (Helliwell et al., 2006; Searle et al., 2006).

Before vernalization, the expression of *FLC* requires *FRIGIDA (FRI)* that maintains sufficient levels of *FLC* expression to repress flowering during fall in winter-annual accessions (Michaels and Amasino, 1999; Sheldon et al., 1999). It has been reported that FRI directly associates with Cap binding protein 20 (CBP20), a component of the nuclear cap-binding complex, and binds to the 5' end of the mRNA sequentially. Loss of CBP20 resulted in the decreased level of *FLC* mRNA coupled with the increased proportion of unspliced transcripts of *FLC*, indicating that the activation of *FLC* expression by FRI occurs through nuclear CBC with the RNA-binding mechanism (Geraldo et al., 2009). There are other group of genes that functions in the activation of *FLC* by FRI. FRI-LIKE 1 (FRL1), FRI ESSENTIAL (FES1), SUPPRESSOR OF FRI4 (SUF4), and FLC EXPRESSOR (FLX) form a FRI transcription activator complex (FRI-C) to recruit these proteins to *FLC* chromatin with chromatin-modifying complexes (Choi

et al., 2011). Also, the allelic variation at *FRI* is likely to be the primary determinant of various flowering response to ambient temperature in nature (Balasubramanian et al., 2006).

Other genes that are involved in the activation of *FLC* before vernalization have been reported. *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING (PIE1)*, a gene that encodes a member of the ISWI class of ATP-dependent chromatin-remodeling proteins, is required for the activation of *FLC* (Noh and Amasion, 2003). *Arabidopsis* homologs of trxG protein Trithorax(TRX), *ARABIDOPSIS TRITHORAX1, 2 (ATX1, ATX2)* (Alvarez-Venegas and Avramova, 2001; Baumbusch et al., 2001) are other known activators of *FLC*. ATX1 contains a SET domain that has both histone binding and histone methyltransferase (HMT) activity (Rea et al., 2003; Katsani et al., 2001). The mutations of these activator genes lead to early flowering, corresponding to a reduction of *FLC* transcription level (Noh and Amasino, 2003; Pien et al., 2008).

Vernalization triggers the reduction of *FLC* transcript via a mitotically stable epigenetic repression (Sung and Amasino, 2004). Using forward genetics approaches, a group of mutants that shows insensitivity to vernalization, leading plants to flower very late even after the vernalization treatment, have been found and studied for their function in the vernalization pathway. These genetic screens have revealed the following genes: *VERNALIZATION 1(VRN1), VRN2, VERNALIZATION INSENSITIVE 3 (VIN3), VRN5/VIN3-LIKE 1 (VIL1)*, and *atPRMT5* (Bastow et al., 2004, Gendall et al., 2001, Greb et al. 2007, Schmitz et al., 2008, Sung et al., 2006). All mutants of the above genes fail to stably repress *FLC* transcription in response to vernalization. Among these genes, *VIN3* displays a unique expression pattern during cold. The expression of *VIN3* exhibits the vernalizing cold-specific induction pattern, and *VIN3* quickly disappears upon the exposure to the following warm temperature (Sung and Amasino, 2004). The transient

expression pattern of *VIN3* during vernalization suggests that VIN3 is essential to trigger the molecular events that stably repress *FLC* during vernalization. VIL1 was identified as a VIN3-interacting protein by a yeast two hybrid screen, and is a member of the *VIN3* gene family (Sung et al., 2006). *vin3* and *vil1* mutant both result in a vernalization-insensitive phenotype and prevent *FLC* from the repression by blocking the accumulation of repressive histone modifications from *FLC* chromatin (Sung et al., 2006; Greb et al., 2006). A Polycomb Repressive Complex 2 (PRC2)-like complex was identified in *Arabidopsis*, and this complex contains VRN2, CURLY LEAF (an Enhance of *zeste* homolog), SWINGER (an E(z) homolog) and FERTILIZATION-INDEPENDENT ENDOSPERM. This complex interacts with VIN3 and is involved in the early stages of the *FLC* repression by vernalization (Wood et al., 2006).

The repression of *FLC* expression by the cold-induced VIN3 and PRC2 is maintained after the vernalization. In *Drosophila*, the maintenance of the suppression by PRC2 requires other repressive complex known as Polycomb Repression Complex 1(PRC1) (Cao et al., 2002; Levine et al., 2004). However, the *Arabidopsis* genome does not carry any PRC1 component homologs (Hsieh et al., 2003), indicating that other protein that is not a component of PRC1 functions in the maintenance of *FLC* repression after the vernalization. The study of *Arabidopsis HETEROCHROMATIN PROTEIN1 (HP1)* homolog, *LIKE HETEROCHROMATIN PROTEIN1 (LHP1)* exhibited that LHP1 is required to maintain the repressive state of *FLC* after the vernalization (Sung et al., 2006; Mylne et al., 2006). The association of LHP1 with *FLC* chromatin increases, and maintains after the vernalization. LHP1 is involved in silencing euchromatic genes as well as playing a role similar to that of PRC1 in other species (Mylne et al., 2006).

1.4 Histone modifications at *FLC* chromatin

The epigenetic regulation of genes modulates chromatin structure in response to environmental stimuli. An important mechanism of these regulations is the covalent modifications of the histone protein, including histone methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation (Berr et al., 2012). The protein or the protein complex that established the histone modifications are called "writers". After the histone modifications are generated, they become the signals recognized by other proteins called "readers". The readers then recruit additional proteins to spread the histone modification to the neighboring chromatin or to remove the pre-established histone modifications. The proteins that remove the histone marks are called "erasers" (Chi et al., 2010). Chromatin modifiers often include all of three types of proteins, writers, readers, and erasers. The combinatorial work of chromatin modifiers generates the dynamics of histone modifications in response to the environmental stimuli.

Histone modifications can associate with the activation or the repression of gene expression. For example, di- and tri-methylation of lysine (K) residues of the histone H3 at the position 4 (H3K4me2 and H3K4me3) are related to the actively expressed genes. Similarly, tri-methylation of histone H3 lysine 36 (H3K36me3) is also linked to the activation of genes. These are often referred as the activation marks on chromatin. On the other hand, di-methylation at histone H3 lysine 9 (H3K9me2) and tri-methylation at histone H3 lysine 27 (H3K27me3) are associated with the silenced genes. These are the repressive marks on chromatin. The activation marks and the repressive marks play the antagonistic roles in the regulation of gene expression and establish the balanced state at chromatin to mediate fine-tuned regulations (Greer and Shi., 2012; He et al., 2010; Yang et al., 2014).

The level of the *FLC* transcription is maintained high to prevent plants from flowering in the first growing season and sets a vernalization requirement in plants. Consistent with the high level of *FLC* expression before the vernalization, *FLC* chromatin is highly enriched with two activation marks, H3K4me3, and H3K36me3, before vernalization. ARABIDOPSIS TRITHORAX-LIKE1 (ATX1), ATX2, and ATXR7 have been characterized as the methyltransferases for H3K4 that promote the *FLC* expression by increasing the level of H3K4me3 at *FLC* chromatin (Pien et al., 2008; Tamada et al., 2009). Other *Arabidopsis* genes that are homologs of the yeast PAF1 complex components *PAF1* and *CTR9*, *EARLY FLOWERING 7 (ELF7)* and *ELF8*, are essential for the high *FLC* expression, and relate to the enrichment of H3K4me3 at *FLC* chromatin (He and Amasino, 2005, He et al., 2004). The mutations in the genes involved in the deposition of H3K4me3 at *FLC* resulted in early-flowering phenotypes with the decreased level of H3K4me3 enrichment at *FLC* chromatin before vernalization (Alvarez-Venegas et al., 2003; He et al., 2004; Pien et al., 2008; Saleh et al., 2008; Zhang and van Nocker, 2002). The level of H3K4 methylation is controlled not only by histone methyltransferases but also by histone demethylases (Liu et al., 2010; Klose and Zhang, 2007). The human lysine-specific demethylases 1 (LSD1) has three homologs in *Arabidopsis*, which are FLOWERING LOCUS D (FLD), LSD1-LIKE1 (LDL1), and LDL2, and they are all FLC repressors (He et al., 2004; Liu et al., 2007; Jiang et al., 2007). In *fld* mutant, the level of H3K4me2 and H3K4me3 are both up-regulated in the central region of *FLC* chromatin (Liu et al., 2007; Yu and Michaels, 2010; Jiang et al., 2009). The mutants of LDL1 and LDL2 also display the increased expression of *FLC* through the methylation of H3K4 (Jiang et al., 2007).

H3K36me3 is accumulated in gene bodies of the highly expressed genes as an indication of the active transcription (Vakoc et al., 2006; Roudier et al., 2011). The

enrichment patterns of H3K36me3 are slightly different from that of H3K4me3, another histone active mark, displaying a sharp peak at the promoter region with a mild accumulation across the gene body region (Yang et al., 2014). H3K36me3 is one of the histone marks displaying the interesting dynamics during vernalization. The accumulation of H3K36me3 is gradually decreased at *FLC* chromatin, coinciding with the increases of another repressive mark, H3K27me3 during vernalization. The change of H3K36me3 at *FLC* chromatin is quite distinctive from that of genes in other species. However, the similar pattern of H3K36me3 was observed in other *Arabidopsis* genes (Yang et al., 2014). In analysis of *Arabidopsis* genome, this pattern of H3K36me3 over the genic region was again found, and was different from the pattern of H3K36me3 in yeast and mammals (Roudier et al., 2011), indicating that the role of H3K36me3 in *Arabidopsis* is a bit different from that in other species. The profiles of H3K36me3 and H3K27me3 changes at *FLC* chromatin during vernalization exhibit the anti-correlations: decreasing H3K36me3 with increasing H3K27me3. This pattern suggests that H3K36me3 antagonizes H3K27me3 in *Arabidopsis* (Yang et al., 2014). SET DOMAIN GROUP 8 (SDG)/ EARLY FLOWERING IN SHORT DAYS (EFS) has been reported as the specific methyltransferase for H3K36 and H3K4 at *FLC* (Shafiq S et al., 2014; Rao B et al., 2005; Zhao Z et al., 2005; Ko JH et al., 2010; Tang X et al., 2012). The global levels of H3K36me3 significantly decrease in *sdg8* mutant accompanied with the increase of H3K27me3 (Yang et al., 2014), indicating the antagonizing effect between H3K36me3 and H3K27me3.

Although the level of H3K27me3 is inversely correlated with the level of H3K36me3, H3K27me3 itself is the important indicator of the repressive chromatin and is regulated by the multiple proteins. The protein that catalyzes H3K27me3 had been first reported in *Drosophila melanogaster*. Polycomb Repressive Complex 2 (PRC2) is a

complex that includes Extra Sex comb (ESC), Enhancer of zeste (E(Z)), Suppressor of zeste 12 (SU(Z)12), a p55 in *Drosophila* and catalyze tri-methylation of H3K27 *in vivo* (Muller et al., 2002, Ringrose and Paro, 2004; Ketel et al., 2005). Homologous genes of all components of the PRC2 complex are present in *Arabidopsis* genome, including three E(Z) homologs, CURLY LEAF (CLF), MEDEA (MEA), and SWINGER (SWN), three Su(z) 12 homologs, FERTILIZATION-INDEPENDENT SEED2 (FIS2), EMBRYONIC FLOWER2 (EFM2), and VERNALIZATIOM2 (VRN2), five p55 homologs, MULTICOPY SUPPRESSOR OF IRA (MSI) 1-5, and one homologs of Esc, FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) (Pien and Grossniklaus, 2007; Liu et al., 2010; Guitton and Berger, 2005; Hennig et al., 2005). The E(Z) homologs, including CLF, MEA, and SWN, form the PRC2 complexes and catalyze H3K27me3 in *Arabidopsis* (Kohler et al., 2003; Wang et al., 2004; Derkacheva and Hennig, 2014). Three distinct PRC2 complexes are involved in the development of plants; FIS-PRC2 complex, EMF2-PRC2 complex, and VRN2-PRC2 complex. The FIS-PRC2 complex is composed of MEA, FIS2, FIE, and MSI1. This complex functions in female gametophyte and seed development (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999; Kohler et al., 2003; Pien and Grossniklaus, 2007; Bemer and Grossniklaus, 2012). The EMF2-PRC2 complex is consist of EMF2, FLC, FIE, and MSI1 and functions in repression of floral transition. EMF2, CLF, and FIE participate in silencing the floral repressor *FLC* and *FT*, accompanied by the decrease in H3K27me3 at *AGAMOUS (AG)*, *AGAMOUS LIKE 19 (AGL19)*, *FLC* (Jiang et al., 2008; Liu et al., 2010). The *clf* mutant results in early flowering, curled leaves and the poor development of floral organs. The components in this complex exhibit the redundancy. SWN exhibits the redundancy with MEA and CLF. The double mutants, *swn; mea* and *swn; clf*, mutants result in stronger phenotypes than those in *swn*, *mea*, and *clf* single mutant with the great decrease of

H3K27me2/3 (Chanvivattana et al., 2004; Makarevich et al., 2006; Wang et al., 2006; Liu et al., 2010). The VRN2-PRC2 complex is consist of SWN/CLF, VRN2, FIE, and MSI1, triggering the spreading of H3K27me3 across *FLC* chromatin to maintain the repressed state of *FLC* during vernalization (Bastow et al., 2004; Finnegan and Dennis, 2007; De Lucia et al., 2008). The VRN2 requires the VIN3 for the proper vernalization response in plants through a direct interaction (Wood et al., 2006). The defect in either protein compromises the vernalization response followed by up-regulation of *FLC* after vernalization. VIN3 is one of the members of the plant-specific protein family that contains both the plant homeodomain (PHD) and the fibronectin 3 (FNIII) domains. VIN3 is considered as the key regulator of vernalization response by binding to regions of the promoter and the first intron of *FLC* (Sung and Amasino, 2004). VIN3 mRNA is present only during vernalization whereas VRN2 mRNA is constitutively expressed. Besides, VIN3 is co-immunoprecipitated with VRN2 (Wood et al., 2006; De Lucia et al., 2008). In *vin3* and *vrn2* mutants, H3K27me3 is not enriched at *FLC* chromatin, and *FLC* expression maintains high during vernalization (Bastow et al., 2004; Sung and Amasino, 2004). Another VIN3-like protein, VIL1/VRN5, is also required for vernalization response and VIL1 was identified from a yeast two-hybrid screen that was designed for VIN3-interacting proteins (Sung et al., 2006) and *vil1* mutant showed the reduced vernalization response (Grab et al., 2007).

The mutants that showed the decreased H3K27me3 at *FLC* chromatin also showed the decreased the methylation of H3K9 (H3K9me) (Bastow et al., 2004; Sung and Amasino, 2004). H3K9me is also important as a repressive mark in heterochromatin (Schwartz and Pirrotta, 2007). Whereas VIN3 and VRN2 participate in both the methylation of H3K9 and H3K27, VRN1 is involved in only H3K9 methylation during and after vernalization. VRN1 encodes a DNA-binding protein containing two plant-

specific B3 domains (Bastow et al., 2004; Sung and Amasino, 2004). Another protein, LHP1/TFL2 is an *Arabidopsis* homolog of human HP1 (Gaudin et al., 2001; Kotake et al., 2003), that is responsible for the formation and maintenance of heterochromatin structure by binding to the methylated H3K9 (Maison and Almouzni, 2004).

1.5 Modifications of H3K4me3 at *FLC* chromatin

Histone H3K4 methylation plays a major role in the regulation of gene transcription in eukaryotes. The state of methylation of histone H3K4 is dynamically controlled by H3K4 methyltransferases and demethylases. The lysine residue of histone H3 can be mono-, di-, and tri-methylated (Dou et al., 2006; Shilatifard, 2008). Among the three methylated states, H3K4me3 is correlated with the active gene expression and this histone mark is recognized by the evolutionarily conserved ATP-dependent chromatin remodeling units, leading the targeted region to the transcriptional activation (reviewed in Ruthenburg et al., 2007). The methylation of H3K4 is established by the Complex Proteins Associated with Set1 (COMPASS), that was found in *Saccharomyces cerevisiae* and contains a Set1 H3K4 methyltransferase (Miller et al., 2001). Complexes similar to COMPASS have been identified in other species. In human, COMPASS-like complexes including human SET1 complex and MLL1 complex were reported and are capable of catalyzing H3K4 methylation and activating target genes (Shilatifard, 2008). The core components of the COMPASS-like complexes are evolutionarily conserved and consisted of four proteins: a relative of yeast Set1 protein (methyltransferase) with three structural elements of Ash2, RbBP5, and WDR5 (Shilatifard, 2008). The core complex composed of these four proteins reconstituted *in vitro* displayed the specific methyltransferases activity on H3K4 (Dou et al., 2006). Another complex that contains the RNA Polymerase II-Associated Factor 1 (PAF1) also regulates the enrichment of H3K4me3 at target

chromatin. Mammalian PAF1-containing complex consists of six subunits (Paf1, Cdc73, Leo1, Ctr9, Rtf1, and Ski8) (Zhu et al., 2005; Kim et al., 2010). As an elongation factor in transcription, the Paf1-containing complex was shown to facilitate elongation (Chen et al., 2009). The Paf1-containing complex plays an essential role in the acquisition of transcription-associated histone modifications including the methylation of H3K4, H3K36, and H3K79, as well as H2B ubiquitinations (Krogan et al., 2003; Cao et al., 2015; Mbogning et al., 2013). These two complexes, COMPASS and PAF1, physically associate to coordinate the transcription of target genes (Krogan et al., 2003).

As stated above, the level of *FLC* transcription is tightly linked to the H3K4 methylation in *Arabidopsis*. Two classes of proteins, the yeast SET1 class and a class of *Drosophila melanogaster* Trithorax (Trx) are the proteins that catalyze the methylation of H3K4 (Smith et al., 2004; reviewed in Shilatifard, 2008). The proteins in both classes contain the SET1 or SET1-related domains in *Drosophila* (Trx), and ten proteins were identified in *Arabidopsis* as putative H3K4 methyltransferases based on the similarity of Trx or Set1 domain in the structures (Springer et al., 2003; Jacob et al., 2009). Based on the SET domain and the other conserved domain that contains the plant homeodomain (PHD) finger within the structure, five proteins were designated ARABIDOPSIS TRITHORAX (ATX) 1-5 (Alvarez-Venegas and Avramova, 2001; Baumbusch et al., 2001). The remaining five proteins were designated ARABIDOPSIS TRITHORAX-RELATED (ATXR)1-4, and 7. Most of these proteins contain the Trx domains, except ATXR7 which has a full SET domain along with an RNA recognition motif in Set1 (Reviewed in Avramova, 2009). The rapid-flowering phenotypes were observed typically in the mutants of the putative H3K4 methyltransferase genes. *atx1* mutant exhibits a rapid flowering accompanied by the defect of floral organs (Alvarez-Venegas et al., 2003; Pien et al., 2008). *atx1* mutant also shows the reduced *FLC* expression with reduced levels of

H3K4 methylation. *atx1* and *atx2* double mutant exhibit a rapid flowering in the presence of *FRI* (Pien et al., 2008), indicating the redundant function in the transcriptional activation of *FLC*. However, ATX1 and ATX2 might carry different biochemical functions (Saleh et al., 2008). Other *Arabidopsis* homolog of a component of human H3K4 methyltransferase complexes, WDR5a, also exhibits a rapid flowering in the mutant along with the reduced levels of *FLC* expression and H3K4me3 enrichment at *FLC* chromatin. However, a homolog of WDR5a, WDR5b, doesn't show the flowering-related phenotype in the mutant (Jiang et al., 2009). Similarly, the loss of ATXR7 causes the rapid flowering and the effect of loss of ATXR7 becomes greater when atxr7 was introduced into atx1 mutant, displaying a rapid flowering. This result indicates that ATXR7 and ATX1 are redundant each other. Also, the level of H3K4me3 is substantially reduced in *atx1 atxr7* double mutant. Interestingly, the function of ATXR7 is related to that of the Paf1 complex (Tamada et al., 2009). The two *Arabidopsis* homologs of the yeast PAF1 complex components, *ELF7* and *ELF8* (He and Amasino, 2005), are required for high *FLC* expression with H3K4me3 enrichment at *FLC* chromatin. The lesions in *ELF7* and *ELF8* lead early flowering. Other *Arabidopsis* genes that were identified as the relatives of PAF1 are *VERNALIZATION INDEPENDENCE 3 (VIP3), VIP4, VIP5* all display the early flowering with the reduced H3K4me4 level at *FLC* chromatin (Zhang et al., 2003; Zhang and van Nocker, 2002; He et al., 2004).

The demethylation of the methylated H3K4 is also essential for the regulation of genes and chromatin structures. The methylation state of histone proteins is the result of coordination of methyltransferases and demethylases (Klose et al., 2006a; Klose et al., 2006b; Shi et al., 2004). The reversibility of histone methylation *in vivo* has been discovered later compared to other covalent forms of histone modification. The amine oxidase LSD1 was the first histone demethylases discovered for demethylation of the

mono- and di-methylated H3K4 through an FAD-dependent oxidation reaction (Shi et al., 2004). However, this type of demethylase lacks the ability to remove methyl groups from the tri-methylated H3K4. This observation led the studies to seek for other histone demethylases responsible for the demethylation of the tri-methylated H3K4 (Shi et al., 2004). Subsequently, a family of JmjC domain-containing proteins was characterized as histone demethylases that can demethylate any states of histone lysine methylation at several different sites in yeast and animals (Klose et al., 2006b; Agger et al., 2007; Cloos et al., 2006; Whetstine et al., 2006).

FLD, LDL1, and LDL2 are homologs of human LSD1 and are known as *FLC* repressors (He et al., 2003; Liu et al., 2007; Jiang et al., 2007). FLD reduces both the levels of H3K4me2 and H3K4me3 in the central region of *FLC* chromatin with the decrease of *FLC* expression (Liu et al., 2007). The LDL1 and LDL2 also reduce the H3K4 methylation at *FLC* chromatin followed by repression of floral transition (Jiang et al., 2007). *Arabidopsis* genome encodes twenty JmjC domain-containing proteins, and several JmjC domain-containing demethylases were characterized as regulators of flowering time control (Lu et al., 2010; Searle et al., 2010; Yang et al., 2010; Jeong et al., 2009). Two JmjC domain-containing demethylases have been reported as H3K4-specific demethylases (Yang et al., 2012a; Yang et al., 2012b).

1.6 Demethylases that function at *FLC* chromatin

In 2004, the first histone lysine demethylase (KDM): human Lysine-Specific Demethylases 1 (LSD1)/ KDM was discovered (Shi et al., 2004). LSD1 was identified in the histone demethylase complex displaying the activity for the mono- and di-methylated H3K4 (Shi et al., 2004; Pedersen and Helin, 2010; Mosammaparast and Shi,

2010). LSD1 contains three major domains: an N-terminal SWIRM (Swi3p/Rsc8p/Moira) domain, C-terminal AOL (amine oxidase like) domain, and a central protruding Tower domain (Stavropoulos et al. 2006). The AOL domain contains an FAD-binding subdomain forming a catalytic center (Binda et al., 2002). LSD1 catalyzes the demethylation of the H3K4 using the reduction of FAD to FADH2 (Anand and Marmorstein, 2007). Because of this characteristic, the tri-methylated lysine 4 of H3 could not be the substrate for amine oxidases. By contrast, JmjC domain-containing demethylases are iron (II) alpha-ketoglutarate dependent oxygenases and can hydroxylate alkyl groups directly through a hydroxyl radical, which allows the demethylation of the tri-methylated H3K4. Therefore, the JmjC domain-containing demethylases require iron (II) and alpha-ketoglutarate as cofactors (Lan et al., 2008).

Arabidopsis genome encodes 3 LSD1-type demethylases and 21 JmjC-domain containing demethylases. FLD, LDL1, and LDL2 are homologs of LSD1, and they function in repression of *FLC* through demethylation of H3K4 and DNA methylation at *FWA* accompanied by repression of *FLC*, respectively (Jang et al., 2007). The 20 JmjC-domain-containing demethylases are divided into five distinct groups according to sequence similarities, including KDM4/JHDM3, KDM5/JARID1, JMJD6, KDM3/JHDM2 and the JmjC domain-only group. Each group proteins target specific histone lysine residues at different methylation states, showing significant variations in specificity (Fig 2) (Lu et al., 2008; Reviewed in Luo et al., 21014). JMJ11/ELF6 and JMJ1/REF6 are the proteins in KDM4 group. ELF6 is a repressor in the photoperiod pathway and REF6 represses *FLC* in flowering time regulation (Noh et al., 2004). Interestingly, REF6 was reported as an H3K27 demethylase, which means *FLC* is not likely to be a direct target of REF6 (Lu et al., 2011). JMJ14 has been reported as a demethylase for all three states of methylated H3K4, but mostly H3K4me3-specific

(Yang et al., 2012). The overexpression of JMJ15 results in an early flowering with the reduced level of H3K4me3 at *FLC* chromatin. However, the loss of JMJ15 does not affect flowering, indicating the redundancy of JMJ15 with other JmjC domain-containing demethylases (Yang et al., 2012). JMJ18 is another JmjC domain-containing demethylase affecting *FLC* expression through the level of H3K4me3. JMJ18 is predominantly expressed in phloem companion cells and promote the floral transition in *Arabidopsis* by binding to *FLC* chromatin and demethylating H3K4me3 (Yang et al., 2012).

1.7 Acetylation and Deacetylation of *FLC*

Histone acetylation and deacetylation are important post-translational modifications that are correlated with the gene activation and the deactivation. Transcriptionally active genes are often associated with the hyperacetylated histones, whereas inactive genes are linked with hypoacetylated histones (Hbbes et al., 1998; Sterner and Berger 2000; Boycheva et al., 2014). The levels of histone acetylation are controlled by two antagonistic enzymes, histone acetyltransferase (HAT) and histone deacetylases (HDAC), acting on the ε-amino group of lysine residues of histone proteins. The common acetylation targets are H3 lysine (K) residues 9,14, 18 and 23, and H4 lysine (K) residues 8, 12, 16 and 20 (Fuchs et al., 2006). The addition of acetyl groups on the targets, mediated by HATs, neutralizes the positive charge of histone tails and decreases the affinity of histones for DNA (Kurdistani and Grunstein 2003). By this mechanism, the acetylation opens up the chromatin to allow the access of transcription factors and RNA polymerases to the DNA (Mustakov et al., 1998; Puig et al., 1998). Whereas, Hypoacetylation mediated by HDACs has an opposite effect on the chromatin, increases the negative charge of DNA leading more tightly binding of DNA to the histones. Consequently, hypoacetylation of histones is associated with the repression of

gene expression (Hebbes et al., 1988; Chen and Pikkard 1997; Chua et al., 2003). To sum up, HATs and HDACs interact with many the co-activator or co-repressor complexes respectively to regulate the expression of target genes post-transcriptionally (Utley et al., 1998; Gonzalez et al., 2007).

The *Arabidopsis* HATs are grouped into the four classes based on the homology between yeast and mammalian HATs, that are GCN5-related N-acetyltransferase (GNAT), MOZm Ybf2/Sas2 and Tip60 (MOZ), p300/CREB-binding protein (p300/CBP), and TATA-binding protein (TBP)-associated factor (TAF1) (Servet et al., 2010). One of the GNAT-type HATs, GENERAL CONTROL NONDEREPRESSIBLE 5 (GCN5), has been characterized as an acetyltransferase with the catalytic activity to the acetylated histone H3K14, H3K9, and H3K27 at the target gene promoters (Vlachonasios, 2003; Earley et al., 2007). GCN5 is involved in the terminal flower production and the abiotic stress related to temperature in *Arabidopsis* (Cohen et al., 2009; Hu et al., 2015; Mao et al., 2006; Pavangadkar et al., 2010).

CBP/p300-like genes (HAC genes) have been found in plants (Pandey et al., 2002). The *Arabidopsis* HAC1 was demonstrated to possess an HAT1 activity (Bordoli et al., 2001). One of the functions of this HAC gene family is to regulate flowering time in *Arabidopsis*. The transcription level of *FLC* increases substantially in *hac1* mutant compared to wild-type (Deng et al., 2007). HAC family genes are regulating flowering time in the autonomous floral pathway by negatively affecting the expression of *FLC* (Han et al., 2007).

In *Arabidopsis*, 20 Histone deacetylases (HDACs) have been characterized, and these proteins can be categorized into three groups based on phylogenetic analysis. The first type is homologous to the yeast RPD3/HDA1 (Reduced Potassium Deficiency 3) that is the biggest groups of HDACs (Lusser et al., 1997). The second type is the HD-

tuins that is only present in plants (Wu et al., 2000; Dangl et al., 2001). The third type is homologous to the yeast Sir2, which is a nicotinamide adenine dinucleotide (NAD)-dependent enzyme, showing distinctive structure from other two group proteins (Frye 2000; Pandey et al., 2002). (Fig 3)

The RPD3 type HDACs consist of 12 putative members (Pandey et al., 2002). All members of this group share the histone deacetylase domain and can be sub-grouped into three classes. The HD-tuin type HDACs are plant-specific HDACs that were found first in maize (Lusser et al., 1997). In *Arabidopsis*, four HD proteins were reported; HDT1-HDT4 (Wu et al., 2000; Dangle et al., 2001). The last type, Sir2 (silent information regulator 2) HDACs are a bit different from other two groups regarding the structure and functions. Unlike other HDACs, Sir-HDACs are inhibited by trichostatin A (TSA) or sodium butyrate (Jung et al., 1997). *Arabidopsis* genome encodes two Sir proteins, SRT1 and SRT2, in this group.

Several deacetylases function in the flowering pathways in *Arabidopsis*. HDA6 controls flowering time through the interaction with FLD (Yu et al., 2011). Increased levels of the histone H3 acetylation and H3K4me3 at *FLC* chromatin, were observed in both *hda6* and *fld* mutant. Therefore, similar delayed flowering were shown in *hda6*, *fld*, and double mutant. Another RPD3 class HDAC, HDA5, is also involved in flowering time control by repressing *FLC* and *MAF1* expression. Interestingly, HDA5 forms a complex with HDA6, FLD, and FVE (Luo et al., 2015) indicating that these deacetylases might have additional functions through the interaction. There is a deacetylase mutant that showed early flowering, *hda9* (Kang et al., 2015). HDA9 controls the flowering in independent of the *FLC*-mediated pathway, targeting *FT* through the deacetylation of *AGL19* (Kang et al., 2015). However, the functions of deacetylases in the flowering pathway in *Arabidopsis* are still unclear and need to be studied further.

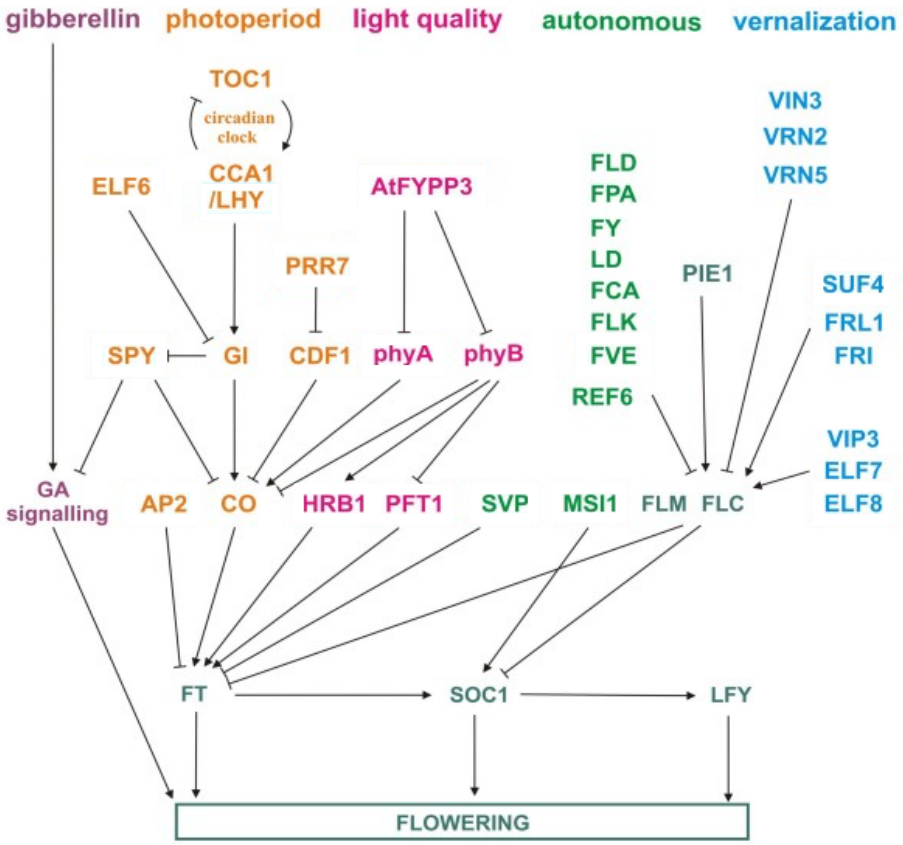

Figure 1. Multiple flowering pathways in *Arabidopsis*.

A summarized chart displaying *Arabidopsis* flowering pathways, including genes with relationships in each pathway. (modified from Katriina Mouhu *et al*., 2009). Five independent pathways are present above, gibberellin, photoperiod, light-quality, autonomous, and vernalization pathway. Some key components are shared by multiple pathways; CO is regulated by mostly components in photoperiod pathway as well as by those in the light-quality pathway; FLC is regulated by components of autonomous pathways before the exposure to cold and vernalization pathway component when vernalization.

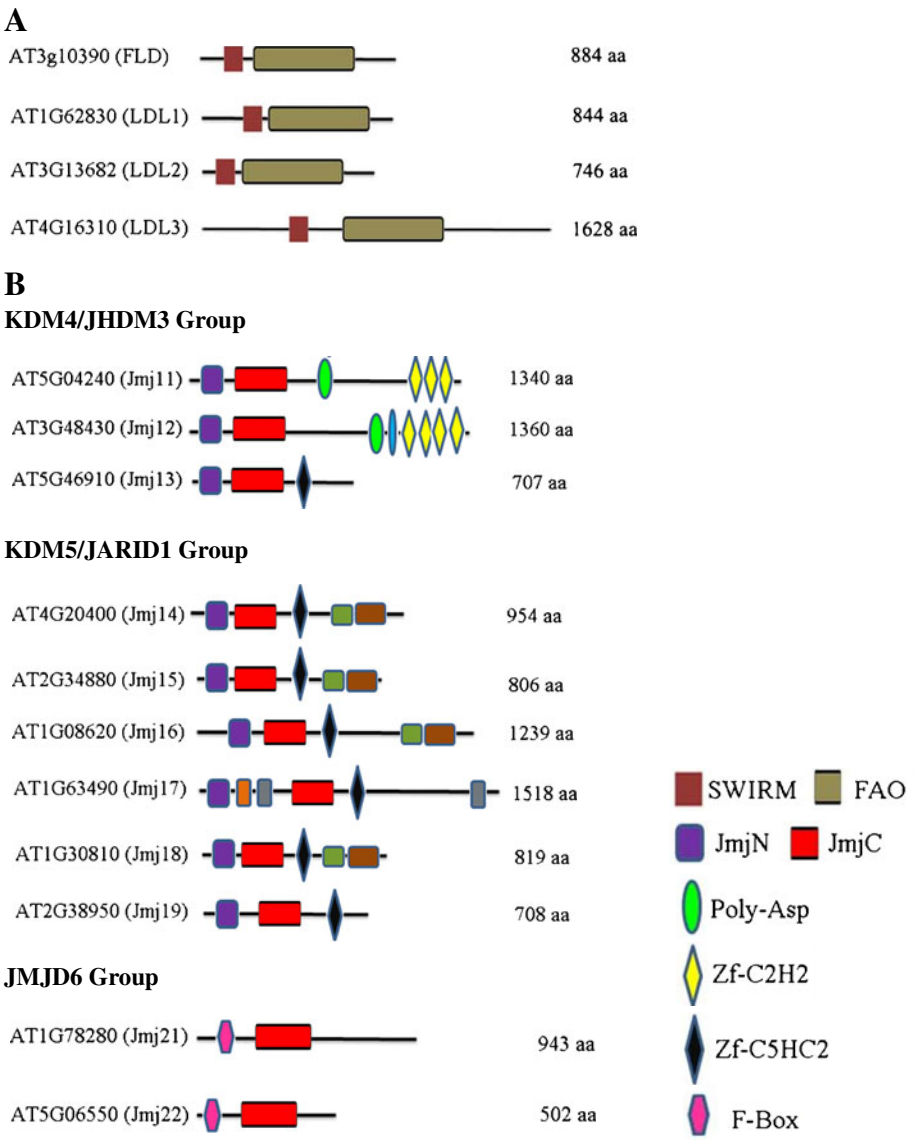

Figure 2. Comparison of the domain structure of histone lysine demethylases in *Arabidopsis*.

(A) Lysine demethylase1/ lysine-specific demethylase1 (KDM1/LSD1) type demethylases. (B) Three groups of Jumonji C (JmjC) domain-containing proteins. KDM4/JDHM3 group, KDM5/JARID1 group, and JMJD6 group. (modified from Luo et al., 2014)

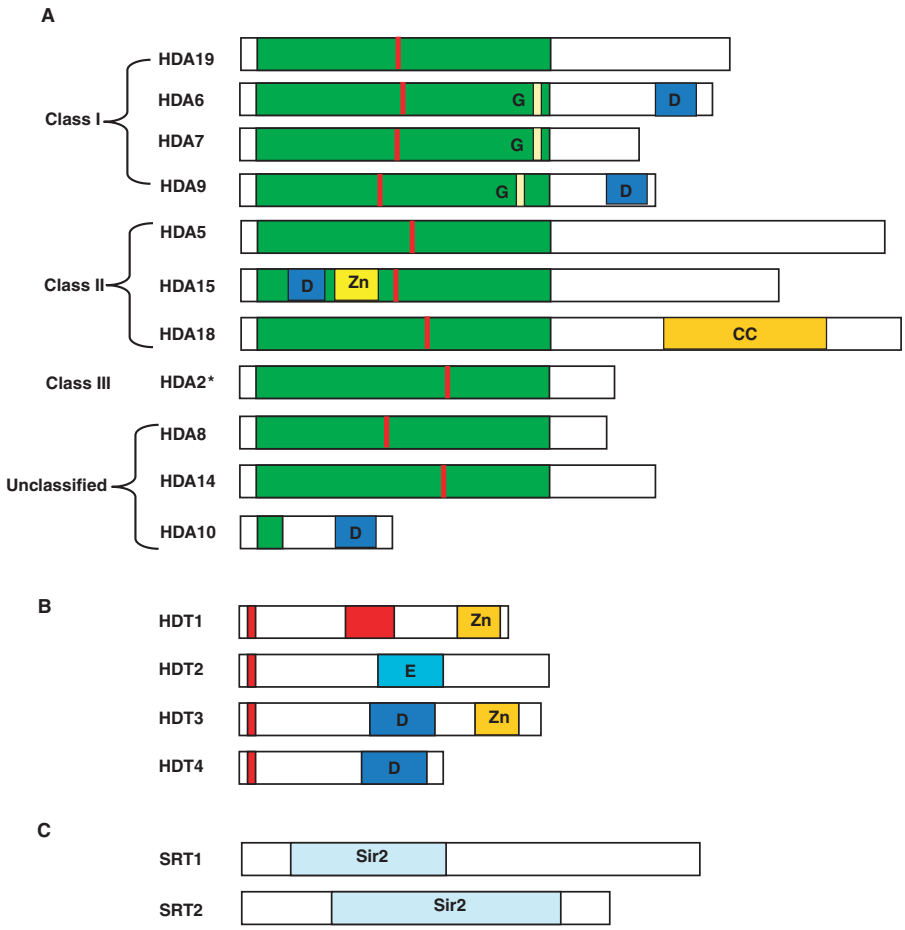

Figure 3. Comparison of domain structure of histone deacetylases in *Arabidopsis*

(A) RPD3-like family HDACs. Green boxes and red regions indicate the conserved HDACs domain and the histone deacetylases activity sites, respectively. G, D, and E represent glycine-rich, aspartate-rich and glutamate-rich regions, respectively. CC represents a coiled-coil domain, and Zn represents a zinc finger domain. (B) HD-tuin HDACs. The small red boxes at the N-terminus represents the conserved EFWG region (functions repressive). The big red box in the HDT1 represents an acidic region required for repression. (C) Sirtuin HDACs. Blue boxes represent the Sir2 domain (modified from Hollender and Liu, 2008).

Chapter 2. Two novel JmjC domain-containing demethylases function at *FLC* chromatin during vernalization

2.1 Introduction

In most plants, the transition from the vegetative phase to the reproductive phase is a critical event in the life cycle. The prolonged cold of winter is one of the important determinants for plants to initiate flowering in the following spring. In biennials and winter annuals, the flowering is typically blocked in the first growing season and is overcome by the following cold winter. This cold-mediated initiation of flowering is called vernalization (Chouard, 1960; Bernier et al., 1981). In *Arabidopsis*, vernalization results in the suppression of the floral repressor, FLOWERING LOCUS C (FLC) (Michaels and Amasino, 1999), and the vernalization-mediated repression of *FLC* is stably maintained mitotically even after returning to warm temperature (Lee et al., 2000; Helliwell et al., 2006; Searle et al., 2006; Michaels and Amasino, 2001). By vernalization, *FLC* chromatin undergoes various histone modifications such as methylation of histone H3 Lys 9 (H3K9) and Lys 27 (H3K27) as well as H3 deacetylation (Bastow et al., 2004; Sung and Amasion, 2004). *VERNALIZATION INSENSITIVE 3(VIN3)*, a PHD-finger domain protein, is induced by vernalization and involved in the repression of *FLC* by multiple histone modifications (Sung and Amasino, 2004). While the increase in these repressive histone marks, the active histone marks including tri-methylation of Histone H3 Lys 4 (H3K4me3) and tri-methylation of Histone H3 Lys 36 (H3K36me3) (Sung et al., 2006; Greb et al., 2007; Yang et al., 2014) gradually decrease by vernalization.

The dynamics of various methylations of histone proteins is determined by a series of histone methyltransferases and demethylases (Liu et al., 2010; Klose and Zhang, 2007). The amine oxidase LYSINE-SPECIFIC DEMETHYLASE 1 (LSD1) is the

histone demethylase characterized in human that shows the demethylase activity on H3K4me2 and H3K4me1 (Shi et al., 2004). However, LSD1 demethylase is unable to remove methyl-groups from tri-methylated lysines, implying the presence of other histone demethylases functioning on tri-methylated lysines residues (Shi et al., 2004). JmjC domain-containing protein family was characterized as histone demethylase that exhibits the ability to reduce all states of histone lysine methylation at several specific sites in yeast and animals (Klose and Zhang, 2007; Mosammaparast and Shi, 2010; Klose et al., 2006). In Arabidopsis, more than 20 JmjC domain-containing genes have been identified, and they function in various biological processes, including flowering time, circadian clock regulation, BR signaling, and more (Noh et al., 2004, Lu et al., 2011, Yang et al., 2012, Yang et al., 2012a, Yang et al., 2012b).

Some JmjC domain-containing proteins have been studied as their roles in flowering time control (Lu et al., 2011, Yang et al., 2012, Yang et al., 2012a, Yang et al., 2012b). JMJ14 is a member of JARID/KDM5 and was reported as the H3K4me3 demethylase involved in flowering time regulation through the repression of floral genes, including *FLOWERING LOCUS T (FT), APETALA (AP1), SUPPRESSOR OF CO OVEREXPRESSION 1 (SOC1)* and *LFY* (Lu et al., 2010). Other two proteins from JARID/KDM5 group, JMJ15 and JMJ18, were characterized as the H3K4me3-specific demethylases and they function in the repression of *FLC* chromatin (Yang et al., 2012a, Yang et al., 2012b). However, no JmjC domain-containing histone demethylases that affect vernalization has yet been found.

In this study, we characterized JMJ16 and JMJ19, two JmjC domain-containing histone H3K4 demethylases, for their functions in the vernalization response. The loss of JMJ16 and JMJ19 resulted in the delayed flowering even after the vernalization treatment and results in the incomplete repression of *FLC* chromatin by vernalization. JMJ16 and

JMJ19 are enriched at *FLC* chromatin by vernalization and are responsible for the demethylation of H3K4me3 by vernalization. Besides, JMJ16 and JMJ19 physically interact with VIN3, the key regulator of *FLC*. Therefore, our results suggest that JMJ16 and JMJ19 are two novel H3K4me3 demethylases that function in the vernalization pathway and their functions are associated with the cold-induced VIN3 protein to repress *FLC* by vernalization.

2.2 Material and Methods

2.2.1 Plant materials and growth conditions

The *Arabidopsis* (*Arabidopsis thaliana*) Columbia-0 (Col-0) was used as the wild-type in this study. *jmj15* (Gabi_663C11), *jmj16* (Salk_029530C), *jmj18* (Sail_861_F02), *jmj19* (Salk_025269C), *vin3-1* were used in this study. Standard growth conditions were 22ºC under illumination with white fluorescent light. The photoperiodic cycle was 16 hours light/ 8 hours dark (long days) or 8 hours light/ 16 hours dark (short day). For vernalization treatments, seeds were surface sterilized, placed on agar-solidified germination medium, and grown for 10 days in a 22ºC growth chamber and transferred to 4 ºC for 40 days under short-day photoperiods.

Gene	Locus	Mutant name	Line
JMJ15	AT2G34880	*jmj15*	GABI_663C11
JMJ16	AT1G08690	*jmj16*	SALK_029530C
JMJ18	AT1G30810	*jmj18*	SAIL_861_F02
JMJ19	AT2G38950	*jmj19*	SALK_025269C
JMJ16;JMJ19	AT1G08690;AT2G38950	*jmj16;jmj19*	SAIL_535_F09; GABI_931H03
VIN3	AT5G57380	*vin3-1*	Sung and Amasino, 2004

Table 1 The mutant lines used in this study

2.2.2 RNA extraction and quantitative real-time PCR analysis

Ten-day-old seedlings were harvested for RNA extraction. Total RNA was extracted using the TRIzol Reagent (Thermo Fisher Scientific). Before reverse transcription, total RNA was treated for 30 minutes at 37ºC with RNase-free DNases I (Invitrogen) to eliminate contaminated genomic DNA, and then total RNA (2 to 3 microgram) was used for cDNA synthesis using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR reaction was done using SYBR green dye reaction mixture (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reaction was performed on ViiA 7 Real-Time PCR system (Thermo Fisher Scientific). PCR primers used for quantitative real-time PCR analysis are listed in Table 2. The relative expression level of each gene was normalized using PP2A (AT1G13320) as described previously (Czechowski et al., 2005).

Gene	Name	Sequence (5' to 3')
JMJ15	JMJ15-real-F	GAAGAGATCATGAGACGCAG
	JMJ15-real-R	CTTATGGTTCCAATACTCCAC
JMJ16	JMJ16-real-F	CGTAGCCGGGTTAAGTACAT
	JMJ16-real-R	TCTTACCATCTCCCAGCATC
JMJ18	JMJ18-real-F	GGGAACTCTCAGCTTCTGAG
	JMJ18-real-R	CCTTCTCCATCTTCTTCAAG
JMJ19	JMJ19-real-F	GAGGAAGCTCCTGTGTTC
	JMJ19-real-R	CTGAATTCCAAAGAGCTGAA
FLC	FLC-real-F	GCCAAGAAGACCGAACTCATGTTGA
	FLC-real-R	CAACCGCCGATTTAAGGTGGCTA
FT	FT-real-F	GCTACAACTGGAACAACCTTTGGCA
	FT-real-R	GGCCGAGATTGTAGATCTCAGCAA
PP2A	PP2A-real-F	TATCGGATGACGATTCTTCGTGCAG
	PP2A-real-R	GCTTGGTCGACTATCGGAATGAGAG

Table 2 Primers used for RT-PCR

2.2.3 Chromatin Immunoprecipitation (ChIP) assay

Chromatin Immunoprecipitation (ChIP) was performed as previously reported (Johnson et al., 2002) with some adjustment for each purpose. The samples of 10-day-old seedlings with no vernalization, 20 days/40 days vernalization, or 40 days vernalization followed by 10-days 22ºC growing were used for ChIP analysis. The samples of seedlings including mutants (*vin3, jmj16;jmj19*) and transgenic (35S:: JMJ16-3XFLAG, 35S:: JMJ19-3X-FLAG) plants were treated with no vernalization, 20 days/40 days vernalization, or 40 days vernalization followed by 10-days 22ºC growing were used for ChIP analysis. To elute DNA from immunoprecipitated complexes, phenol: chloroform: isoamyl (Sigma-Aldrich) precipitation followed by Ethanol precipitation was used. Antibodies recognizing H3K3me3 (EMD-Millipore catalog number 07-473, Abcam ab8580), FLAG-M2 (Sigma-Aldrich, F1084) were purchased and used for ChIP assay at a concentration of up to 10 micrograms per reaction. The quantitative real-time PCR reaction was performed using SYBR green dye reaction mixture (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reaction was performed on ViiA 7 Real-Time PCR system (Thermo Fisher Scientific). The levels of enrichment of proteins at each locus are normalized by the enrichment of the protein at PP2A (AT1G13320) locus. PCR primers used for quantitative real-time PCR analysis in ChIP assay are listed in Table 3.

2.2.4 Generation of transgenic plants

cDNAs of *JMJ16* and *JMJ19* were cloned into the pPZP211 vector containing 3 repeats of FLAG tag after the cloning site (cloned vectors by courtesy of Dr.Noh, Seoul Nat'l Univ). Each construct was transformed into plants with Col background. The *Agrobacteria* strain GV3101 and was used to generate the delivering constructs, and two

day-grown transformed *Agrobacterium* was introduced into plants using floral dip methods (Clough and Bent, 1998). All transgenic lines were selected on selective antibiotic media and were confirmed by PCR and Western blot.

Name	Sequence (5' to 3')
FLC-ChIP-1F	CGTGAGTCCGCCCCTGATAGC
FLC-ChIP-1R	GGACCAAACCAAACCTACAAAGACTTTC
FLC-ChIP-2F	CTTAGTATCTCCGGCGACTTGAACC
FLC-ChIP-2R	GCGTCACAGAGAACAGAAAGCTGA
FLC-ChIP-3F	ATGACTTTGTTCCTATTCGTTAAAATTGACAATC
FLC-ChIP-3R	GACAAGTGTTGTGGGATTTTCAATTTC
FLC-ChIP-4F	CTGCTTTGTTTGTTGCAGACCGAAC
FLC-ChIP-4R	CCGATCAAGTTTCTAAACCTAACAACTTC
PP2A-ChIP-F	AGAAAGCTGTGGATTCACTTTGTAGAATTGGTGC
PP2A-ChIP-R	GACAGAGCATGGAAAGGAATCAATAAAATCGACC

Table 3. Primers used for ChIP-qPCR

2.2.5 Flowering time analysis

Seeds on plates were stratified at 4°C for two days before being transferred to a short day growth chamber at 22°C for seven to ten days. In a short day chamber, plates were exposed to 8 hours light/ 16 hours dark per day. Seedlings were then transplanted before cold exposure to be used as a non-vernalized, NV sample. Some seedlings were transferred to 4°C refrigerator (complete with a light cycle set at 8 hours light/16 hours dark per day) to be treated with vernalization. Seedlings on plates were exposed to 40 days of vernalizing cold. Additionally, plates were brought back to the 22°C short day growth chamber for 10 days after 40V. Ten to twelve seedlings of each line of each time points were transplanted to the soil to be used as a 40days-vernalized sample, 40V.

Rosette leaves of the plants were counted, and an average of the number amongst replicates was used to determine the flowering phenotype under each condition and lines.

2.2.6 Co-Immunoprecipitation Assay

Co-immunoprecipitaton (Co-IP) experiments were performed using 10 days-old seedlings with 40 days of vernalization. 0.4~0.5 g materials were collected and ground, and subsequently nuclear proteins were precipitated by extractions in 10ml of Extraction buffer 1 (2mM EDTA, 2mM DTT, 10mM HEPES (pH8.0), 40mM sucrose, 1mM phenylmethylsulphonyl fluoride, and 1x Roche proteinase inhibitors), 1ml of Extraction buffer 2 (10mM HEPES (pH8.0), 10mM $MgCl_2$, 0.5% Triton X-100, 250mM Sucrose, 1mM phenylmethylsulphonyl fluoride, and 1x Roche proteinase inhibitors), and 500ul of Extraction buffer 3 (10mM HEPES (pH8.0), 2mM $MgCl_2$, 0.15% Triton X-100, 1.7M Sucrose, 1mM phenylmethylsulphonyl fluoride, and 1x Roche proteinase inhibitors). Then, pellets were disrupted by sonication and nuclear proteins were recovered in IP-Buffer (IP-B: 1mM EDTA, 10% glycerol, 75M NaCl, 0.05% SDS, 100mM Tris-Cl (pH 7.4), 0.1% Triton X-100, 1mM phenylmethylsulphonyl fluoride, and 1x Roche proteinase inhibitors). Protein extracts were incubated with anti-FLAG M2 (Signa) at 4°C for overnight and added Dynabeads Protein G (Invitrogen) followed by additional incubation at 4°C for 2 hours. Immunoprecipitated proteins were washed three times with IP-B, and collected and subsequently boiled in the 2XSDS-PAGE loading buffer, followed by Western blotting with anti-FLAG (Sigma), anti-VIN3 (Homemade antibody).

2.2.7 Yeast two-hybrid assay

The full-length cDNA sequences of *JMJ* genes, *VIN3* and *VIL1* were separately cloned into the yeast Gateway-compatible Destination vectors, *pDEST32* and *pDEST22*,

to examine the interaction between JMJ proteins and VIN3 protein. The interaction between VIN3 and VIL1 proteins was used as a positive control for the assay. Different combinations of the *pDEST32* and *pDEST22* constructs were transformed into the yeast strain MaV203. The positive strains were selected from a synthetic dropout medium minus Leu and Trp (SD-LT) and then used for growth assay on synthetic dropout medium minus Leu, Trp, His and Ade (SD-LTHA) supplemented with 3m 3-AT. Controls were simultaneously grown on SD (LT) medium.

2.3 Results

2.3.1 H3K4me3 decreases at *FLC* chromatin by vernalization

The repression of *FLC* by vernalization is established by modifications of various histone markers (Sung 2004, Yang et al., 2014) and the dynamics of a repressive histone marker, H3K27me3, at *FLC* chromatin during vernalization has been well studied (Jiang et al., 2008; Angel et al., 2011). However, how the active histone markers at *FLC* are regulated by vernalization remains unclear. One of active histone markers, tri-methylated histone H3 at lysine 4 (H3K4me3) was highly enriched at *FLC* chromatin before the vernalization, and the enrichment was due to the multiple methyltransferases such and ARABIDOPSIS TRITHORAX 1 (ATX1), ATX2, and ATX-RELATED 7 (ATXR7) (Yun et al., 2012). To address whether the repression of *FLC* by vernalization accompanies changes in the level of H3K4me3, we analyzed the level of H3K4me3 at two regions at *FLC* locus by ChIP (Fig 4). Consistent with the previous report (Yun et al. 2012), the enrichment of H3K4me3 at *FLC* around the first exon region (which is indicated with P2) was observed before vernalization, and the enrichment decreased

significantly during vernalization. This region is known regions to be critical for *FLC* regulations (Sung et al., 2006). This result suggested that the decrease in the level of H3K4me3 at *FLC* chromatin is associated with repression of *FLC* during vernalization.

2.3.2 JARID/KDM5 group proteins are demethylases specifically targeting tri-methylated Histone H3 Lysine 4

Two types of histone lysine demethylases have been known in eukaryotes: the lysine demethylase 1/ lysine-specific demethylase1 (KDM1/LSD1) type and the Jumonji C (JmjC) domain-containing demethylases (Lan et al., 2008) (Table1). In Arabidopsis, there are four LSD1 homologs, FLOWERING LOCUS D (FLD), LSD1-like (LDL1), LDL2 and LDL3 (Jiang et al., 2007). FLD, LDL1, and LDL2 were shown the ability to demethylate mono- and di-methylated H3K4 but no ability for tri-methylated H3K4 (Jiang et al., 2007). However, FLD is known to involve in demethylation of H3K4me3 together with RELATIVE OF EARLY FLOWERING 6 (REF6/JMJ12) (Ko et al., 2010).

There are 21 JmjC domain-containing proteins in *Arabidopsis* (Lu et al., 2008). Among the five groups of Arabidopsis JMJ proteins (KDM4/JHDM3, KDM5/JARID1, JMJD6, KDM3/JHDM2, JmjC domain-only groups), KDM5/JARID1 group has been studied as histone demethylases specifically targeting H3K4 as substrates (Table 4). Within the KDM5/ JARID1 group, JMJ15 and JMJ18 have been reported as the H3K4me3-specific demethylases targeting *FLC* chromatin before vernalization (Yang et al., 2012a; Yang et al., 2012b). JMJ16 and JMJ19 have not been reported regarding demethylases activity. However, the structures of JMJ16 and JMJ19 are very similar to JMJ15 and JMJ18, sharing the functional domains JmjN and JmjC domain that are the essential catalytic motifs of JmjC domain-containing histone demethylases (Fig 5A, Fig 5B). JMJ15, JMJ16 and JMJ18 proteins contain the binding residues for alpha-

ketoglutarate (alpha-KG) and Fe (II) while JMJ19 lacks this co-factor binding residues (Fig 5B).

2.3.3 Mutation in JMJ16 and JMJ19 delays flowering under short days with vernalization

In order to search for the demethylase(s) that function(s) in the vernalization pathway, we isolated the T-DNA insertional mutants for four *JMJ* genes (*JMJ15, JMJ16, JMJ18*, and *JMJ19*), and measured the flowering time under short day condition (16h dark/ 8h dark) with and without 40 days of cold treatment. We excluded JMJ14 from our analysis based on the report that JMJ14 directly targets to *FLOWERING LOCUS T (FT)* (Yang et al., 2010). We also excluded JMJ17, based on its structural difference from other proteins in the same group. The flowering time of each single *jmj* mutant was indistinguishable from wild-type plants before and after the vernalization except *jmj15* mutant. *jmj15* mutant showed the delayed flowering before and after the vernalization (Fig 6), but *jmj15* mutant showed normal vernalization response, the accelerated flowering by vernalization. This result led us to examine the possibility of the redundancy among *JMJ* genes. To obtain clues for genes in redundancy for vernalization response, we analyzed the pattern of the relative expression of the KDM5/JARID1 group genes in the course of vernalization. Interestingly, two *JMJ* genes, *JMJ16* and *JMJ19*, showed the vernalization-mediated induction. On the other hand, other related JMJ genes, *JMJ15* and *JMJ18,* showed decreased or steady level of expression during vernalization (Fig 8). Therefore, we chose JMJ16 and JMJ19 as candidate demethylases that may function in the vernalization pathway.

Because none of the single mutants exhibited defects in their vernalization response, we tested the double mutant of *JMJ16* and *JMJ19* (*jmj16;jmj19*) for its vernalization response. *jmj16;jmj19* mutant is a null mutant for *JMJ16* and unable to

generate a full transcript of *JMJ19* (Fig 7). Single *jmj16* and *jmj19* mutant showed similar flowering phenotypes with wild-type while the double mutant *jmj16;jmj19* showed delayed flowering after vernalization (Fig 9).

We next examined the expression profile of the *FLC* in wild-type and the double mutant during vernalization. We collected seedling samples from four time-points of non-vernalized, 20 days vernalized, 40 days vernalized, and 10 days after 40 days of vernalized condition and tested them. *jmj16;jmj19* showed elevated *FLC* expression than wild-type at all tested conditions except non-vernalized condition (Fig 10A). As expected, the expression of *FT* in *jmj16;jmj19* is lower than wild-type during vernalization (Fig 10B). This result implies that the delayed flowering of *jmj16;jmj19* after vernalization associated with the de-repression of *FLC* during vernalization followed by the down-regulation of *FT*, the major downstream target of FLC.

In the vernalization pathway, *FLC* is the main target for the mechanism, although there are *FLC* clade genes, including *MAF1/FLM* to *MAF5*, which are controlled by vernalization. These *FLC* clades also participate in the regulation of flowering through the regulation of *FT* (Ratcliffe et al., 2001; Ratcliffe et al., 2003). Another MADS-box gene, *AGL19*, is also reported to involve in the vernalization-mediated flowering, independent of *FLC* pathway (Schönrock et al., 2006). We wonder whether the loss of JMJ16 and JMJ19 affects the other genes in the vernalization pathway. We analyzed the expression profiles of *FLC* clade genes and *AGL19* during vernalization (three-time points: NV, 40V, and T10). Overall patterns of their expression are similar to wild-type, suggesting that JMJ16 and JMJ19 mainly function in the regulation of *FLC* (Fig 11).

2.3.4 JMJ16 and JMJ19 are involved in H3K4me3 regulation at *FLC* by vernalization

We next measured the H3K4me3 enrichment at *FLC* chromatin in *jmj16;jmj19* and wild-type by vernalization, in which the *jmj16;jmj19* double mutant showed the elevated *FLC* expression and the delayed flowering (Fig 9, Fig 10). Consistent with *FLC* expression, we found that the level of H3K4me3 at *FLC* chromatin are higher in *jmj16;jmj19* during vernalization at the indicated regions (Fig 12). In particular, the reduction in the level of H3K4me3 at *FLC* by vernalization is impaired in the double mutant, indicating that JMJ16 and JMJ19 are responsible for demethylating H3K4me3 at *FLC* chromatin to repress *FLC* expression during vernalization.

Results above suggest that JMJ16 and JMJ19, either or both directly associated with *FLC* chromatin to mediate the demethylation of *FLC* chromatin. To investigate this possibility, first, we created the transgenic plants overexpressing JMJ16-3X FLAG and used them for Chromatin Immunoprecipitation (Due to the lower expression of the JMJ16 protein, we generated the overexpressor lines and used them for ChIP). The *35S:: JMJ16-3XFLAG* transgenic plants showed the flowering phenotype similar to wild-type under long days (Fig 20). An antibody specific to FLAG was used to precipitate chromatins with JMJ16 proteins from wild-type and two independent *35S:: JMJ16-3X FLAG* T1 plants. The JMJ16 was significantly enriched to *FLC* chromatin in transgenic lines. The enrichment mostly occurs at the upstream and the first exon region (P1 and P2) of *FLC* chromatin in all time points of vernalization (Fig 13). This result indicates that JMJ16 associated with *FLC* chromatin and the association might be responsible for the demethylation of H3K4me3 at *FLC* chromatin by vernalization. The association of JMJ19 with *FLC* chromatin was also analyzed by ChIP. The enrichment of JMJ19 at *FLC* chromatin was observed in transgenic plants that express JMJ19-FLAG and not in wild-type plants (Fig 14), indicating that JMJ19 is also associated with *FLC* chromatin.

Collectively, this result is consistent with the functional redundancy of JMJ16 and JMJ19 in the regulation of *FLC* by vernalization.

2.3.5 JMJ16 and JMJ19 interact with cold-induced VIN3

The most upstream regulator in the vernalization pathway that has been identified is VIN3, a PHD finger protein that interacts with PRC2 components to repress *FLC* during vernalization (Sung and Amasino, 2004; De Lucia et al., 2008; Wood et al., 2006). VIN3 protein is induced by at least 10 to 20 days of cold and repressed to the basal levels when plants are returned to the growth temperature (Sung and Amasino, 2004; Fig 15A). The repression of *FLC* by the cold-induced VIN3 and the PRC2 complex is established through various chromatin modifications including H3K27me3, H3K36me3, H3K9me2 and more (Sung and Amasino, 2004; Kim and Sung, 2013). VIN3 has been considered as the regulator to control other histone modifications occurred at *FLC* chromatin such as the demethylation of H3K4me3 and the acetylation of H3. However, the mechanism about these modifications remains unclear.

To investigate the involvement of VIN3 to the regulation of H3K4me3 at *FLC* chromatin, we performed the ChIP experiment to analyze the level of H3K4me3 in *vin3* mutant with wild-type as controls. The reduction in the level of H3K4me3 at *FLC* chromatin was not observed in *vin3* mutant by vernalization (Fig 15B). This result is similar to that in *jmj16;jmj19* double mutants (Fig 12). The patterns of the H3K4me3 level at P2 region of *FLC* in *vin3* mutant and *jmj16;jmj19* mutant plants were similar during vernalization, showing that the demethylation of H3K4me3 did not occur properly and led the de-repression of *FLC* during vernalization (Fig 12, Fig 15B). This observation led us to address the possibility that VIN3 and JMJ16/JMJ19 function in the same complex to regulate *FLC* expression.

We performed the yeast two-hybrid analysis to test interactions between VIN3 and JMJ proteins (JMJ14, JMJ15, JMJ18, and JMJ19). Among JMJ proteins, only JMJ19 interacted with VIN3 as strong as VIL1 (used as the positive control: Sung et al., 2006) (Fig 16). Unfortunately, we could not test the JMJ16 for the interaction with VIN3 at this time because JMJ16 was not successfully cloned into the yeast vector. Therefore, we analyzed the interaction between JMJ16 and VIN3 proteins later by co-immunoprecipitation (Co-IP) assay using the transgenic plants that express JMJ16-FLAG. As shown in Fig 17, VIN3 were co-immunoprecipitated by JMJ16-FLAG. Our results suggested that JMJ16 and JMJ19 proteins interact to VIN3 protein directly.

2.4 Discussion

In this study, we characterized two novel JmjC domain-containing histone demethylases, JMJ16 and JMJ19, that control H3K4me3 at *FLC* chromatin and are involved in flowering time control by vernalization in *Arabidopsis*. The transcription of *JMJ16* and *JMJ19* were significantly induced in response to cold, and the elevated level of transcription was maintained during vernalization and even when returning to warm temperature (Fig 8), indicating that these two demethylases carry out the vernalization-specific functions. Consequently, *jmj16;jmj19* double mutant showed the delayed flowering when grown under short days after the 40 days of cold treatment (Fig 9) without compromising flowering time in the absence of vernalization treatment. The expression level of the floral repressor, *FLC*, was affected in *jmj16;jmj19* double mutant during vernalization. The vernalization response achieved by repression of the *FLC* transcription by long-term exposure to cold was compromised in *jmj16;jmj19* double mutant, explained by repression of the *FT* transcript, the main target gene of *FLC* (Fig 7).

However, the expression of other genes in the vernalization pathway such as *FLC* clade genes (*MAF1-MAF5*) and *AGL19* was not affected (Fig 11). These results suggest that JMJ16 and JMJ19 function mainly through the regulation of *FLC* and *FT* rather than *MAFs* and *AGL19* in *Arabidopsis*. In addition, JMJ16 and JMJ19 directly interact with VIN3 (Fig 16, Fig 17), implying the demethylation of H3K4me3 by JMJ16 and JMJ19 might require VIN3 to function. Based on these observations, we propose a model for the functions of JMJ16 and JMJ19 at *FLC* during vernalization (Fig 18). In this model, the demethylation of H3K4me3 by JMJ16 and JMJ19 occurs at *FLC* chromatin during vernalization in wild-type, changing the chromatin from the active state to the repressive state. As a result, *FLC* chromatin becomes fully repressed with the decreased H3K4me3 when returning to warm temperature. The absence of JMJ16 and JMJ19 in mutants blocks the demethylation of H3K4me3 at *FLC* chromatin further maintaining the active state of chromatin even with vernalization treatment. The role of VIN3 in this model is yet unclear, but the interaction between VIN3 and JMJ16/JMJ19 suggests that these proteins might form a complex. There are still questions what other genes are controlled by JMJ16 and JMJ19 in the flowering pathway or other unknown mechanisms. In order to search more target genes of JMJ16 and JMJ19, we would consider a RNA-Seq experiment. The comparison of differently expressed genes in the *jmj16;jmj19* double mutant and wild-type would reveal the additional functions of JMJ16 and JMJ19 that we have not found in the previous analysis. In addition, the RNA-Seq experiments using single *jmj16* and *jmj19* mutant are supposed to enlighten the unknown function of JMJ16 and JMJ19 that were overlooked in the previous study due to the indistinctive phenotype of the single mutants.

In a previous report of the functions of JMJ15, the single mutant of *JMJ15* did not exhibit any flowering phenotype. Instead, over-expression of JMJ15 results in accelerated

flowering under long days and short days with the changes in expression of *FLC* and *FT* expression (Yang et al., 2012). In addition, the overexpression of JMJ15 brought the additional phenotypes such as the enhanced salt tolerance and the repression of genes related to the stress tolerance (Shen et al., 2014). Similarly, JmjC-domain only group demethylase, JMJ30, did not display the different phenotypes from wild-type in single mutants but the over-expression of JMJ30 did display the delayed flowering at the elevated temperature condition and significant changes in the regulation of H3K27me3-related genes (Gan et al., 2014).

It is a bit difficult to characterize the independent functions of JMJ16 and JMJ19 when the distinctive phenotype was only observed in the double mutant plant, not in each single mutant. Considering the previous several JMJ demethylases reports mentioned above, we might try to analyze the transgenic plants expressing JMJ16 and JMJ19 for the flowering phenotypes on vernalization and comparison of differentially expressed genes from the wild-type plants. Due to lack of clues for the specific or additional functions of JMJ16 and JMJ19, genome-wide approaches such as an RNA-Seq experiment might be a good start for searching for the genes negatively/positively regulated by overexpression of JMJ16 and JMJ19.

The level of *FLC* expression in *jmj16;jmj19* double mutant during vernalization is higher than in wild-type, but the level of *FLC* in the double mutants still decreased by vernalization (Fig 10A), suggesting that additional redundancy among other JMJ proteins for the vernalization response. VIN3, the main cold-specific regulator of *FLC*, represses the *FLC* transcription by the methylation of H3K27me3 (Sung and Amasino, 2004) and demethylation of H3K4me3 at *FLC* chromatin (Fig 12). The *vin3* mutant does not respond to vernalization and exhibits extremely late flowering phenotype (Sung and Amasino, 2004). Compared to the *vin3* mutant phenotype, the *jmj16;jmj19* double mutant

exhibit weaker phenotype. In winter-annual accessions (containing *FRI*), the loss of each methyltransferases ATX1, ATX2, and ATXR7 accelerated flowering time in plants and the combination of all loss of methyltransferases caused a synergistic effect on flowering. The plants carrying *atx1 atx2 atxr7* mutation in *FRI* background flowered early almost like rapid-cycling lines (Yun et al., 2012). This result indicates that there are multiple methyltransferases act on the same H3K4me3 at *FLC* in *FRI* background plant with redundancy. The slight response to vernalization observed in *jmj16;jmj19* double mutant plants might be due to the existence of the other demethylase(s) in the same flowering pathway. To investigate this hypothesis, we are generating the higher order of *JMJ* mutants including *jmj16;jmj19* as a background. The partial list of the higher order of mutant includes *jmj15;jmj16;jmj19, jmj16;jmj17;jmj19, jmj16;jmj18;jmj19*. These mutants will be tested for the vernalization response in future.

In this study, we observed that the level of H3K4me3 at *FLC* chromatin was reduced during vernalization in wild-type for the repression of *FLC* transcription whereas the reduction was compromised in the *jmj16;jmj19* double mutants (Fig 12). In addition, we have found that JMJ16 and JMJ19 proteins were enriched at the same location of *FLC* chromatin (Fig 13, Fig 14). These results suggested the direct H3K4me3 demethylase activity of JMJ16 and JMJ19 functioning in the vernalization response. Other H3K4-specific demethylases, JMJ14, JMJ15 and JMJ18 have been proved their enzymatic activities by in vitro assays (Jeong et al., 2009; Yang et al., 2010; Yang et al., 2012a, Yang et al., 2012b). JMJ14 has been tested for the demethylase activity of all states of histone H3K4, H3K9me3, and H3K36me3 (Jeong et al., 2009) due to the wide range of histone substrates for demethylation found in other JMJ proteins (JMJ11: Yu et al., 2008; Noh et al., 2004, JMJ12: Noh et al., 2004; Ko et al., 2010; Lu et al., 2011). The results in Jeong et al., 2009 indicate that JMJ14 is an intrinsic H3K4-specific demethylase for all

three states and does not control other histone substrates. JMJ15 was analyzed for its demethylase activity (Yang et al., 2012). Unlike JMJ14, JMJ15 only demethylates tri-methylated H3K4 and showed no effects on H3K4me2 and H3K4me1, indicating more specific function of JMJ15. Interestingly, JMJ18 has displayed the demethylase activity for H3K4me3 and H3K4me2 and no activity for H3K4me1 (Yang et al., 2012). The results in Yang et al., 2012 also indicated that the linker region between JmjN and JmjC domain is important for demethylation of H3K4me2, but no effect on demethylation of H3K4me3. In our study, JMJ16 and JMJ19 are highly conserved proteins in the KDM5/JARID1 group. However, there are small structural differences present in JMJ16 and JMJ19. Especially, JMJ19 protein lacks the co-factor-binding residues that were shared among all KDM5/JARID1 group proteins. Therefore, the further analysis of the demethylase activity of JMJ16 and JMJ19 is essential, and the analysis of enzymatic activity for the each histone substrate should be followed to understand the functions of JMJ16 and JMJ19.

Previously, the JmjC domain-containing proteins have displayed the unique spatial expressions related to their functions. For example, the expression of JMJ14 is restricted in leaves to repress flowering by decreasing the expression of the floral integrators including *FT, SOC1, LFY*, and *AP1* (Jeong et al., 2009; Lu et al., 2010; Yang et al., 2010). JMJ18 is specifically expressed in vascular tissue to target the *FT* for flowering time control and the expression of *JMJ18* is developmentally regulated (Yang et al., 2012). These studies indicate that the organ/tissues in which JMJ proteins express might reveal the information of possible target genes of JMJ proteins. The expression level of *JMJ16* and *JMJ19* in seedlings was relatively low before the vernalization (Fig 19) and became higher during the vernalization (Fig 8). The further study of the tissue-

specific expression of *JMJ16* and *JMJ19* might reveal epigenetic information about the target genes of JMJ16 and JMJ19 as well.

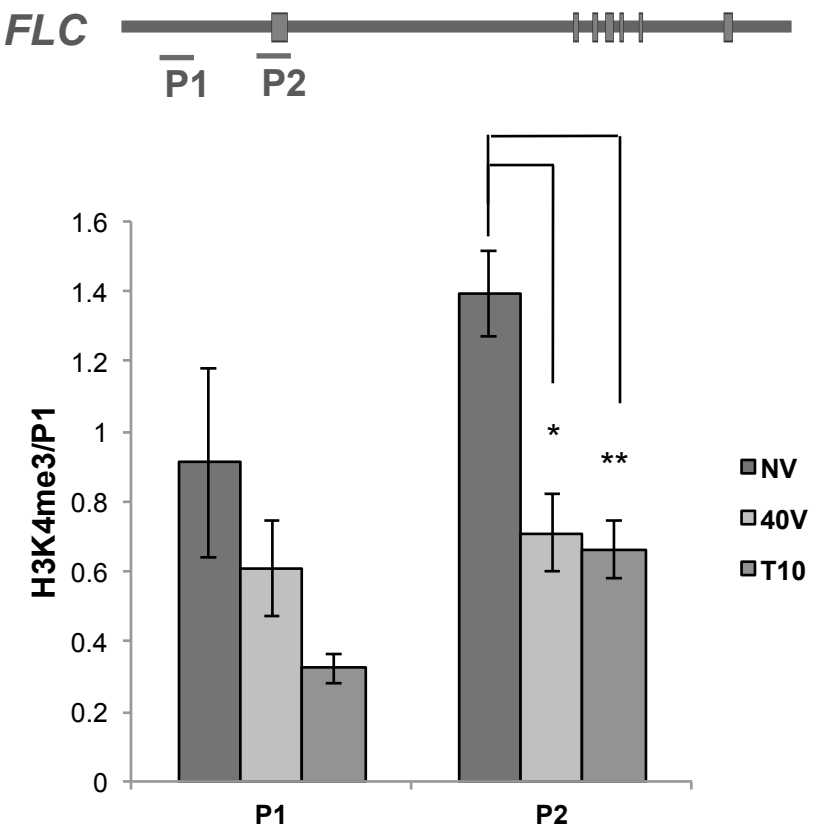

Figure 4. Decreased level of H3K4me3 at *FLC* chromatin during vernalization

(A) A schematic representation of the genomic structure of *FLC* and positions of the primers used in ChIP assays are indicated (P1~P4). (B) The levels of enrichment of H3K4me3 at *FLC* chromatin in *FLC_Col* (WT), during vernalization. Data (mean ± SD of quantitative PCR: n=3). Non-vernalized; NV, 40 days of vernalization; 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10. (two tailed Student's *t*-test, * : $p < 0.05$, ** : $p < 0.01$)

LSD1 homologue	FLD LDL1 LDL2 LDL3	human LSD1 homolog
KDM4/JHDM3 group	JMJ11 JMJ12 JMJ13	JmjC domain-containing protein
KDM5/JARID1 group	JMJ14 JMJ15 JMJ16 JMJ17 JMJ18 JMJ19	
JMJD6 group	JMJ21 JMJ22	
KDM3/JHDM2 group	JMJ24 JMJ25 JMJ26 JMJ27 JMJ28 JMJ29	
JmjC domain-only group	JMJ20 JMJ30 JMJ31 JMJ32	

Table 4. Histone demethylases in *Arabidopsis*

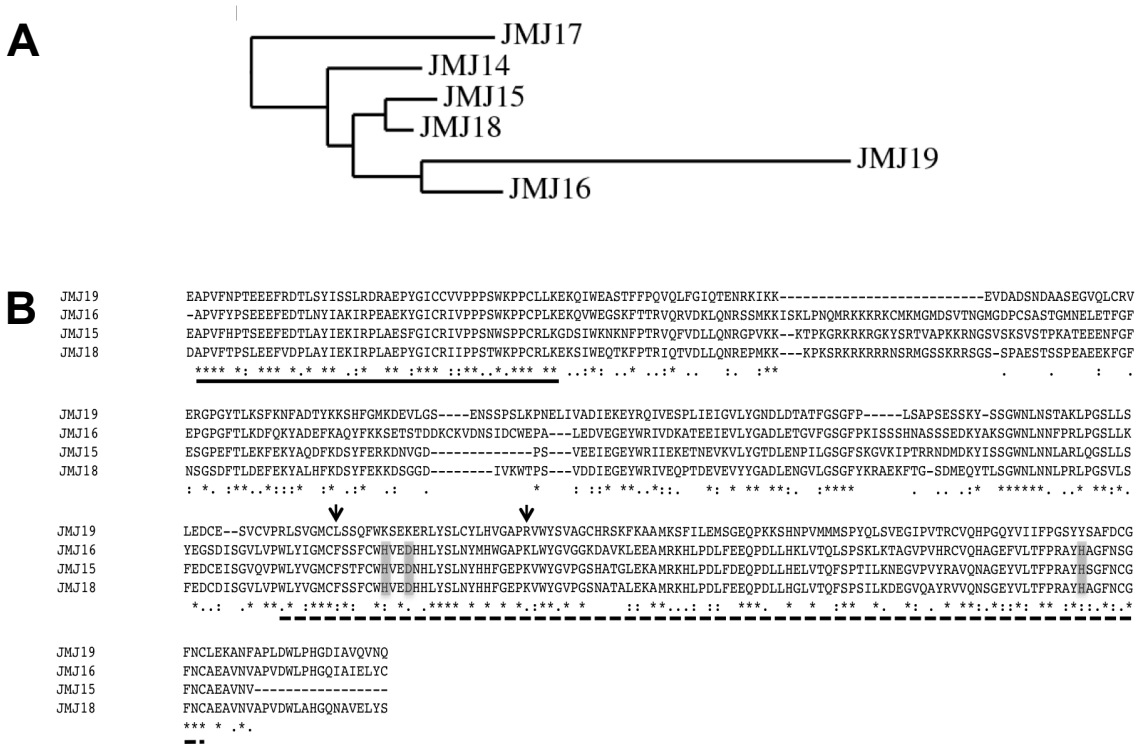

Figure 5. The phylogenetic relationships among KDM5/JARID1 group proteins and specific residues and domains on KDM5/JARID1 proteins

(A) Phylogenic relationships among KDM5/JARID1 group proteins (B) Partial sequence alignment of four closed JARID proteins in *Arabidopsis*. *Solid line*, *dotted line*, *arrows*, and *gray box* indicate JmjN domain, JmjC domain, alpha-Ketoglutarate-binding residues, Fe (II)-binding residues, respectively.

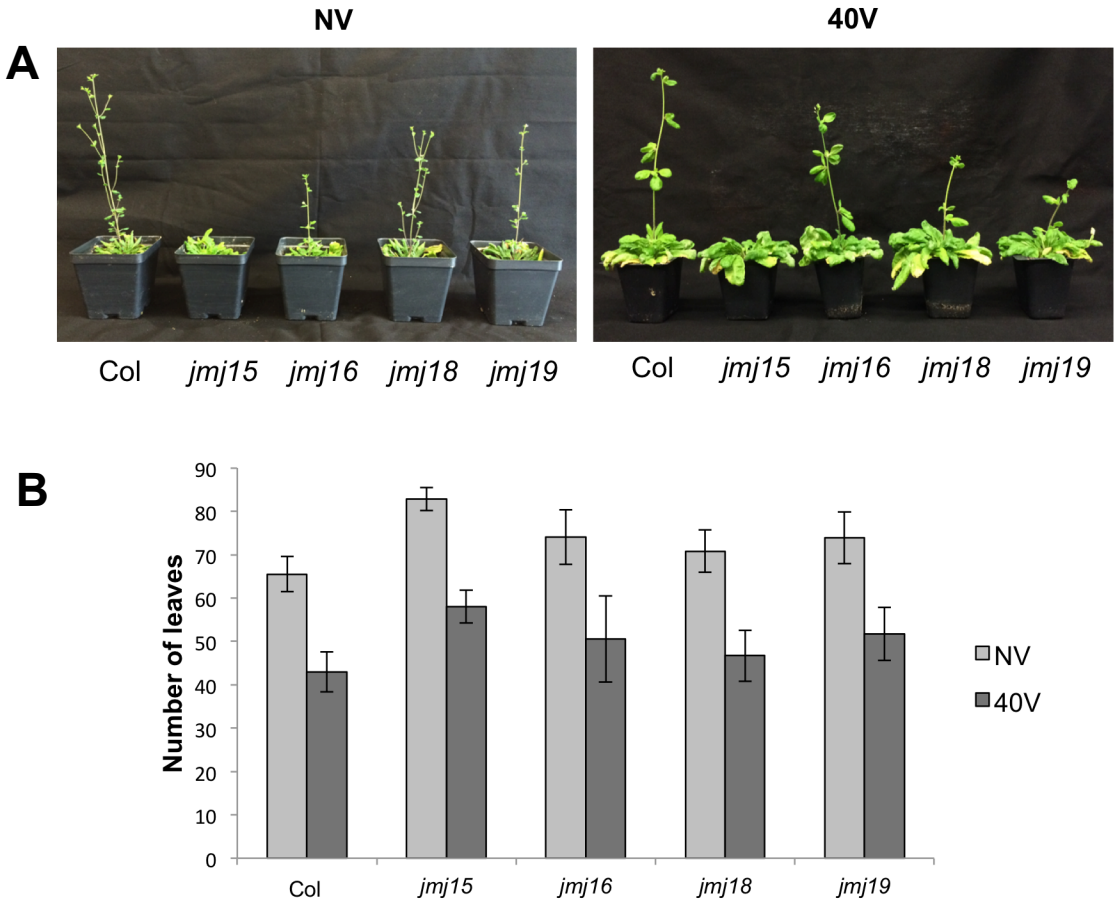

Figure 6. Vernalization responses in single mutant plants of *JMJ* genes

(A) The flowering of wild-type (Col) and the single mutant plants of *JMJ15*, *JMJ16*, *JMJ18*, and *JMJ19* with vernalization treatment (indicated by 40V) and without vernalization treatment (indicated by NV). (B) An average number of leaves at the time of flower in wild-type and each single *JMJ* gene mutants plant.

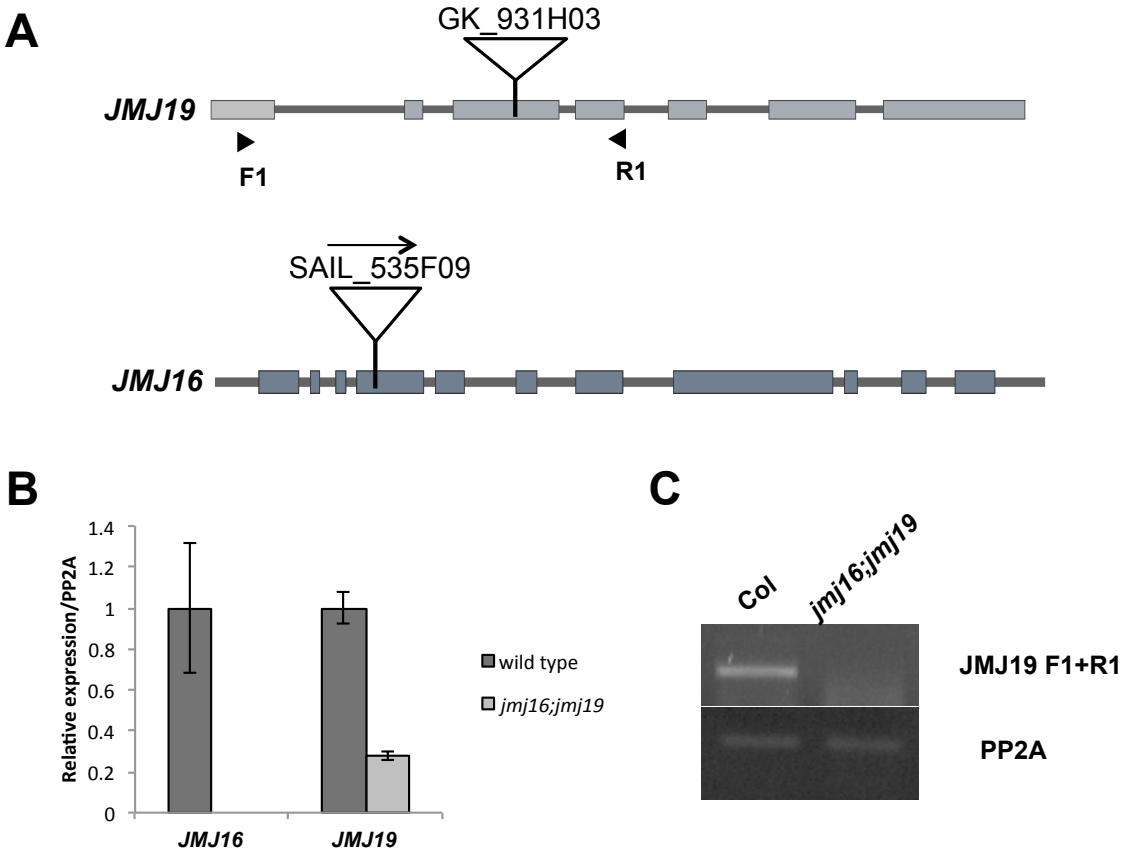

Figure 7. Characterization of *jmj16;jmj19* double mutant

(A) A schematic representation of the genomic structure of *JMJ16* (bottom) and *JMJ19* (top) and positions of the primers used to detect the full transcript of *JMJ19* are indicated (F1 and R1). (B) The levels of *JMJ16* and *JMJ19* full transcripts in WT and *jmj16;jmj19* mutant. (C) The level of *JMJ19* full transcript was not detected in *jmj16;jmj19* mutant in further PCR analysis.

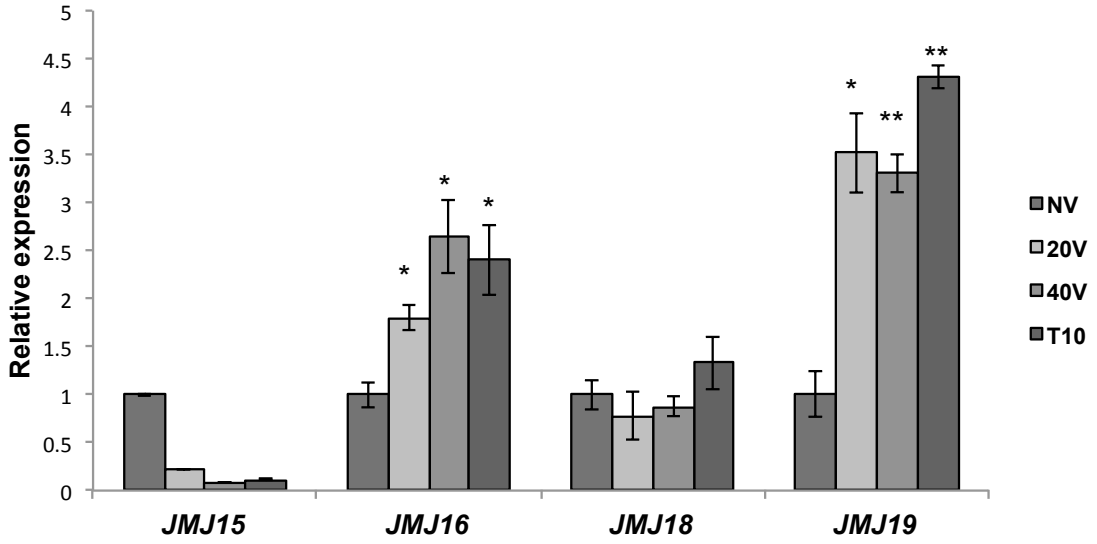

Figure 8. The expression of *JMJ16* and *JMJ19* are increased during vernalization

Relative expression of *JMJ* genes (*JMJ15, JMJ16, JMJ18, and JMJ19*) during vernalization. Non-vernalized; NV, 20, 40 days of vernalization; 20V, 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10. (two tailed Student's *t*-test, * : $p < 0.05$, ** : $p < 0.01$)

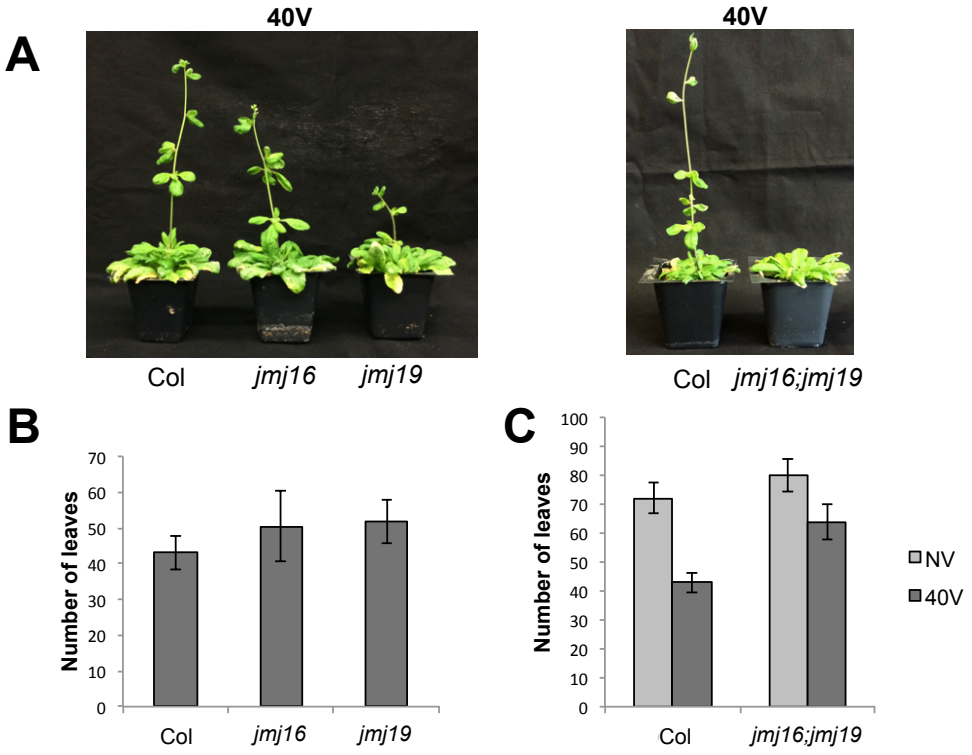

Figure 9. Defect in vernalization responses in *jmj16* and *jmj19* double mutant

(A) The flowering of wild-type (Col) and the single mutant plants of *JMJ16* and *JMJ19* genes and the double mutant of *JMJ16* and *JMJ19* with vernalization treatment (40V). (B) Flowering time of wild-type, *jmj16*, and *jmj19* plant with vernalization treatment. (C) The vernalization responses of wild-type and the *jmj16;jmj19* double mutant plants.

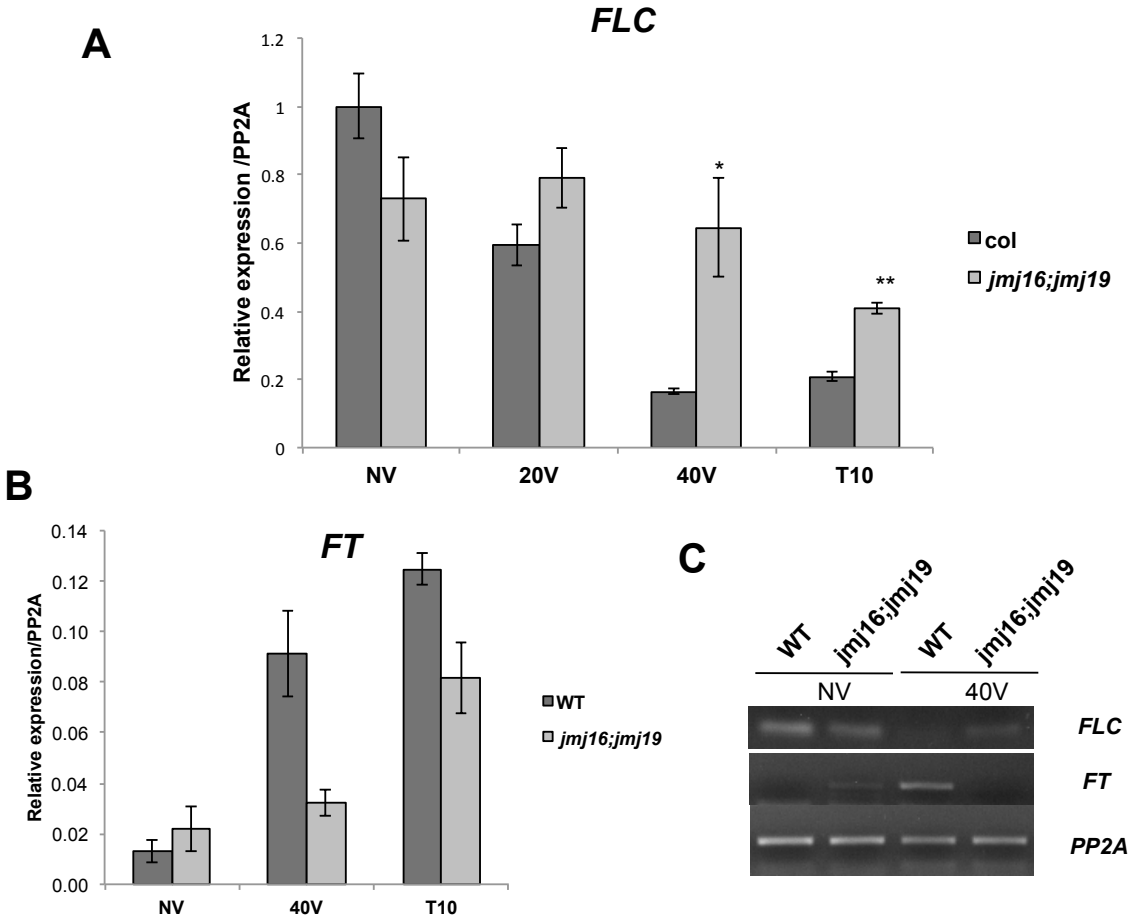

Figure 10. De-repression of *FLC* in the *jmj16;jmj19* double mutant during vernalization.

(A) Relative expression of *FLC* in wild-type and double mutant plant during vernalization. (B) Relative expression of *FT* in wild-type and double mutant plant during vernalization. (C) Relative transcription level of *FLC* and *FT* in wild-type and double mutant from two-time points (NV: non-vernalized, 40V: 40 days of vernalization) by PCR analysis. (two tailed Student's *t*-test, * : $p < 0.05$, ** : $p < 0.01$)

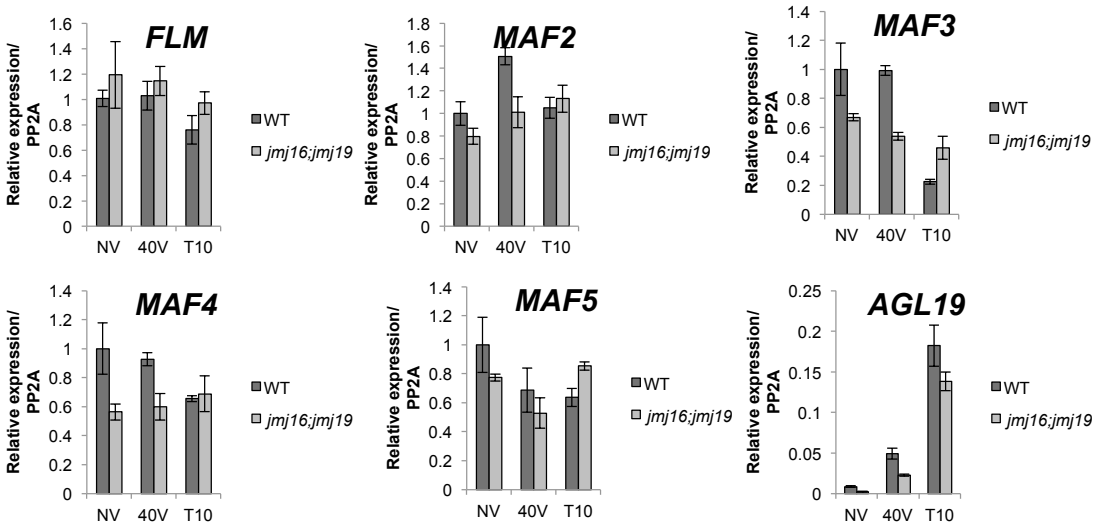

Figure 11. Expression profiles of *FLC* clade genes and *AGL19* in wild-type and the *jmj16;jmj19* double mutant

Relative expression of *FLM*, *MAF2*, *MAF3*, *MAF4*, *MAF5*, *and AGL19* in wild-type and the *jmj16;jmj19* double mutant plant during vernalization. Non-vernalized; NV, 40 days of vernalization; 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10.

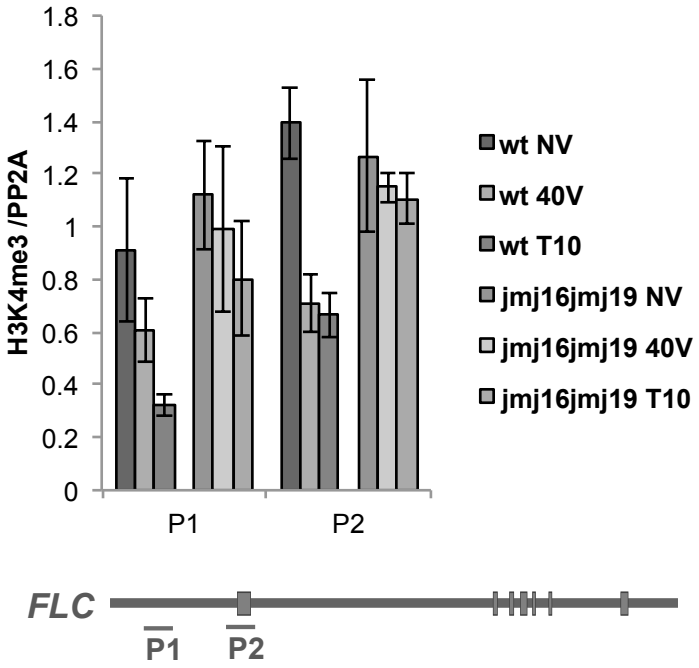

Figure 12. The reduction of H3K4me3 at *FLC* chromatin in wild-type during vernalization is affected by the loss of *JMJ16* and *JMJ19*.

The level of enrichment of H3K4me3 at *FLC* chromatin (P1 and P2 region) in wild-type (WT) and the double mutant plant of *JMJ16* and *JMJ19 (jmj16;jmj19)* during vernalization. Data (mean ± SD of quantitative PCR: n=3). Non-vernalized; NV, 40 days of vernalization; 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10.

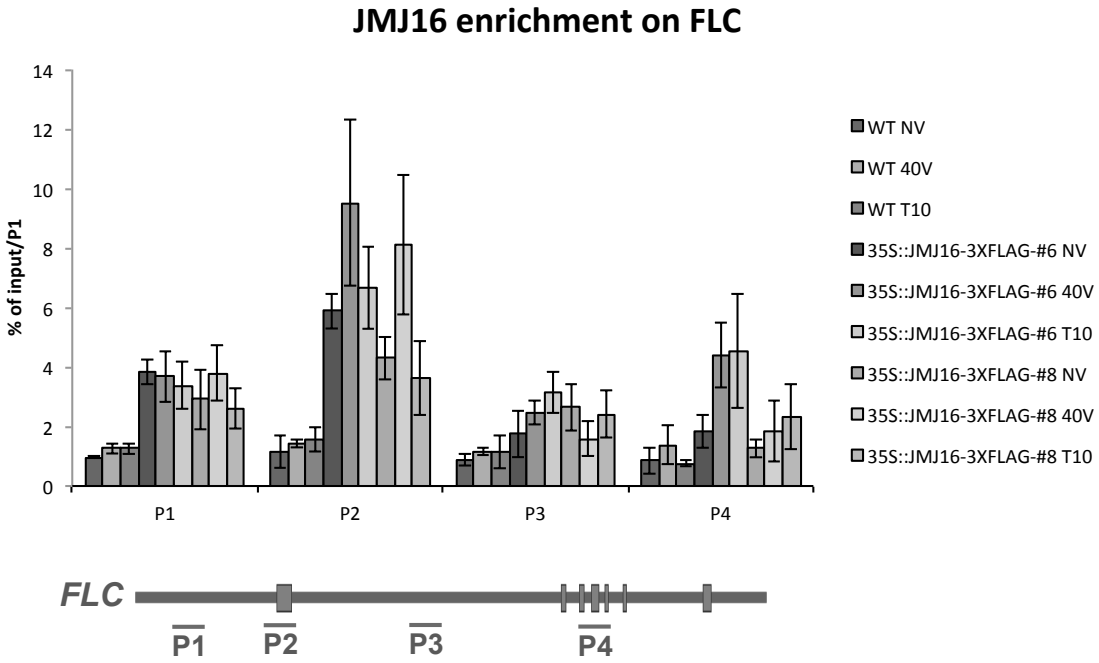

Figure 13. Enrichment of JMJ16-FLAG at *FLC* chromatin in wild-type and two independent JMJ16 transgenic plants.

The level of enrichment of JMJ16-FLAG at *FLC* chromatin in wild-type (WT) and the two transgenic plants that are expressing JMJ16-FLAG during vernalization. The location of primers is indicated by P1~P4. Data (mean ± SD of quantitative PCR: n=3). Non-vernalized; NV, 40 days of vernalization; 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10. (two tailed Student's *t*-test, * : $p < 0.05$)

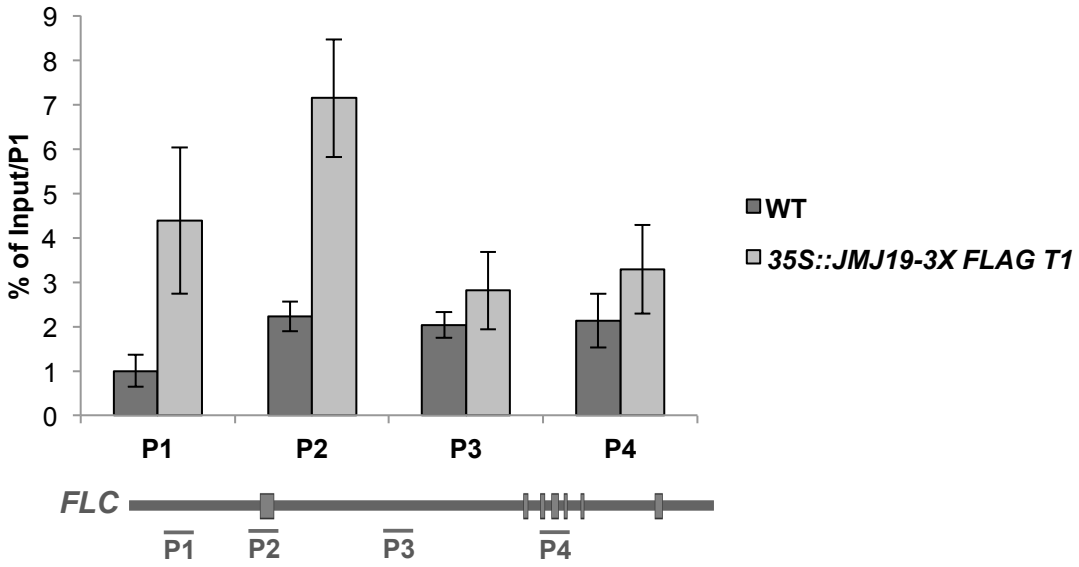

Figure 14. Enrichment of JMJ19-FLAG at *FLC* chromatin in wild-type and JMJ19 transgenic plants.

The level of enrichment of JMJ19-FLAG at *FLC* chromatin in wild-type (WT) and the T1 transgenic plants that are expressing JMJ19-FLAG. The location of primers is indicated by P1~P4. Data (mean ± SD of quantitative PCR: n=3). (two tailed Student's *t*-test, * : $p < 0.05$, ** : $p < 0.01$)

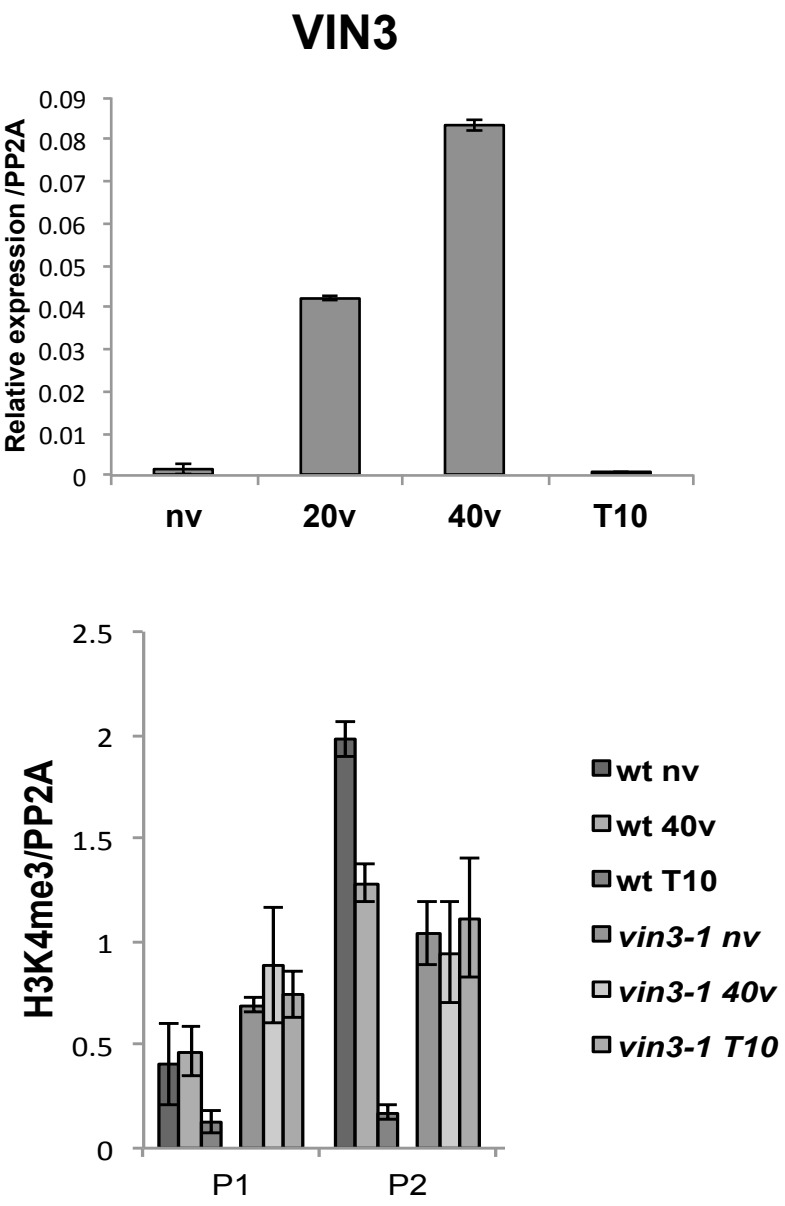

Figure 15. The reduction of H3K4me3 at *FLC* chromatin in wild-type during vernalization is affected by the loss of *VIN3*.

(A) Expression profiles of *VIN3* during vernalization (B) The levels of enrichment of H3K4me3 at *FLC* chromatin in wild-type (WT) and the *vin3 (vin3-1)* mutant during vernalization. Data (mean ± SD of quantitative PCR: n=3). Non-vernalized; NV, 40 days

of vernalization; 40V, and 40 days of vernalization followed by 10 days of normal growth temperature; T10. (two tailed Student's *t*-test, * : $p < 0.05$, ** : $p < 0.01$)

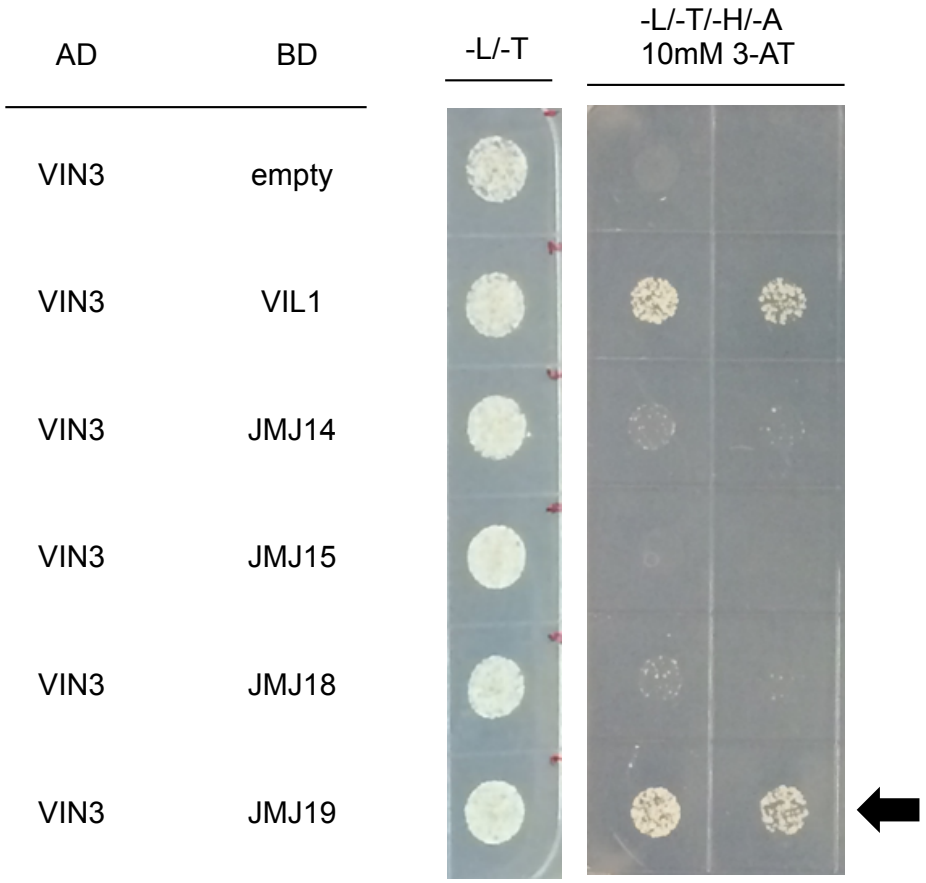

Figure 16. The interaction between JMJ19 and VIN3 was determined by yeast two-hybrid assay

The full-length cDNA sequences of *JMJ* genes were cloned into the yeast vectors *pDEST22* (AD) and *pDEST32* (BD). The direct interaction between JMJ19 and VIN3 was detected by yeast two-hybrid assay. pDEST22: AD, pDEST32: BD.-LT(double drop out; Leu ,Trp),-LTHA (quadruple dropout; Ade, His, Leu, Trp) with 10mM 3-AT(3-Amino-1,2,3-trizole) The interaction of VIN3 and VIL1 was used as a positive control. (Sung et al., 2006).

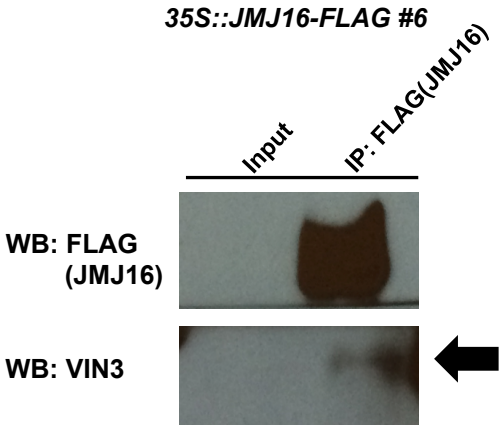

Figure 17. The interaction between JMJ16 and VIN3 was determined by co-immunoprecipitation assay

The full-length *JMJ16–3XFLAG* transgene was introduced into wild-type plants. The transgenic plants expressing JMJ16 with FLAG were used to determine the interaction of the full-length JMJ16 with VIN3 by co-immunoprecipitation. The protein extract for co-IP was isolated from 40 days-vernalization treated seedling in which VIN3 protein was expressed.

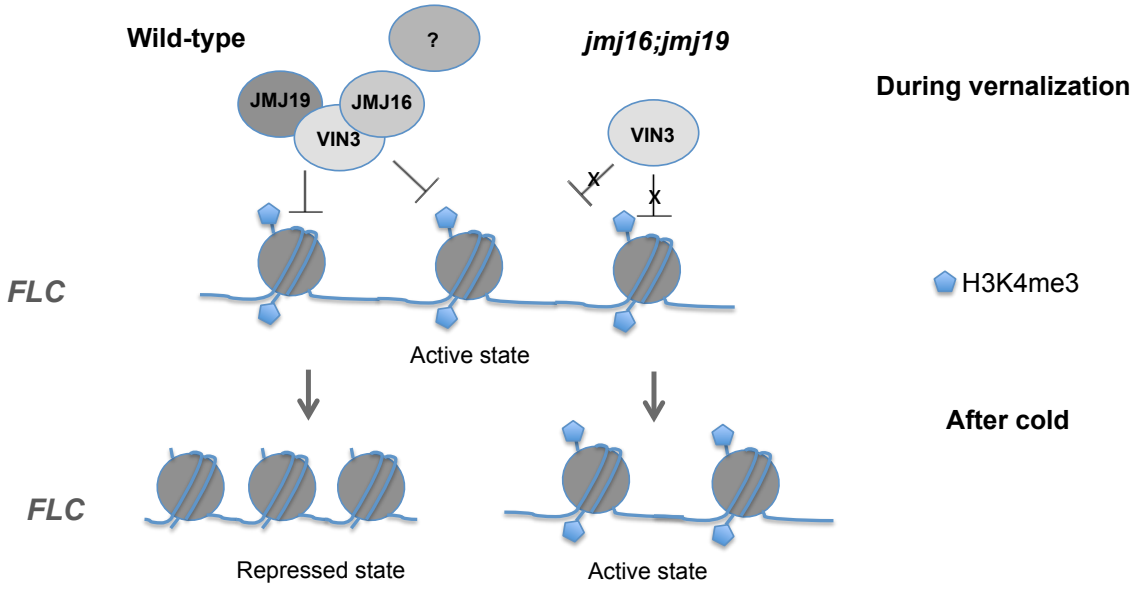

Figure 18. Model for the functions of JMJ16 and JMJ19 associated with VIN3 at *FLC* chromatin during vernalization

The demethylation of H3K4me3 by JMJ16 and JMJ19 occurs at *FLC* chromatin during vernalization in a wild-type plant, changing the chromatin from active state to repressive state. As a result, *FLC* chromatin becomes fully repressed with decreased H3K4me3 when returning to warm temperature. The absence of JMJ16 and JMJ19 in mutants blocks the demethylation of H3K4me3 at *FLC* chromatin further maintaining the active state of chromatin during vernalization.

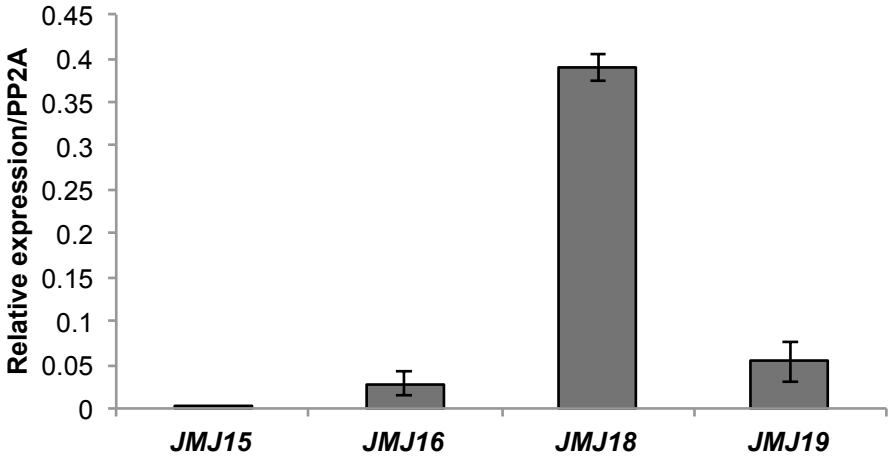

Figure 19. The *JMJ* genes are expressed at a low level, except for *JMJ18*.

The relative expression of *JMJ* genes (*JMJ15*, *JMJ16*, *JMJ18*, and *JMJ19*) in Col-0.

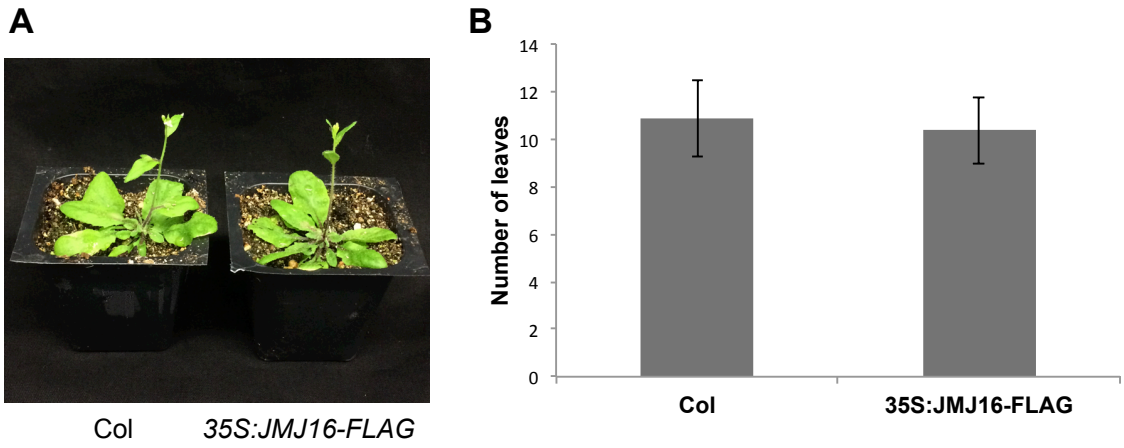

Figure 20. Flowering phenotype of *35S::JMJ16-FLAG* under long day

(A) Flowering of wild-type (Col) and the *35S::JMJ16-FLAG* transgenic plant under long days (B) An average number of leaves at the time of flower in wild-type (Col) and the *35S::JMJ16-FLAG* transgenic plant

Chapter 3. Characterization of two deacetylases involved in flowering under short days

3.1 Introduction

In eukaryotes, epigenetic mechanisms including post-translational modifications of histones play important roles in plant development and responses to the environmental changes through regulation of genes. The multiple residues of histone can be modified by acetylation, methylation, phosphorylation, ubiquitination, and sumoylation by enzymes and chromatin-associated factor (Berger, 2007). These modifications are reversible, and the antagonistic histone-modifying enzymes balance the degrees of modifications. Histone acetylation/deacetylation is tightly linked to the expression of genes that are regulated by counteracting enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC) (Liu et al., 2014). Hypoacetylated histone and hyperacetylated histone are associated with the gene repression and the gene activation, respectively.

HDAC in eukaryotes are grouped into three families based on the phylogenetic distance: reduced potassium dependency-3/histone deacetylase-1 (RPD3/HDA1), histone deacetylases-2 (HD)-tuin, and silent information regulator-2 (SIR2) (Yang and Seto, 2007; Liu et al., 2014). *Arabidopsis* has 18 HDACs that are assigned twelve proteins in the RPD3/HDA1 group, four proteins in HD-tuin group, and two proteins in the SIR2 group. The RPD3/HDA1 group proteins are the most studied HDACs. The proteins in this group are involved in various biological processes including development, reproductive processes, hormone signaling and DNA methylation (Liu et al., 2013; Cigliano et al., 2013; Zhou et al., 2005; Long et al., 2006; Aufsatz et al., 2002; Ryu et al., 2014). The HD-tuin group proteins are plant-specific deacetylases, and four proteins in this group have been reported for their functions in plant development and stress responses (Wu et al., 2000; Zhou et al., 2004; Sridha et al., 2006; Luo et al., 2012). The

SIR group contains two deacetylases, SRT1 and SRT2, which are regulating mitochondrial energy metabolism and cellular dedifferentiation, respectively (Lee et al., 2016; König et al., 2014).

Flowering in *Arabidopsis* is one of the well-known epigenetic mechanisms responding to the environmental cues through histone modifications on genes. The *FLC* chromatin is heavily modified with multiple histone modifications, including histone deacetylation (He et al., 2003; Jiang et al., 2007; Liu et al., 2007). HDA6 controls flowering time by increasing histone H3 acetylation and H3K4 tri-methylations at *FLC* chromatin (Yu et al., 2011). HDA6 interacts with FLD and HDA5 to form an HDAC complex (Luo et al., 2015). The loss of *HDA6* and its interacting proteins in the HDAC complex result in the delayed flowering (Yu et al., 2011; Luo et al., 2012). Unlike HDA6, another deacetylase, HDA9 controls flowering time by targeting *AGAMOUS-LIKE 19 (AGL19)* and *FT* (Kang et al., 2015). The loss of *HDA9* causes early flowering under short days and the increased acetylation level in *AGL19* locus (Kang et al., 2015; Kim et al., 2013). However, the involvement of histone deacetylases in the flowering mechanism remains unclear.

In this study, we characterized the two deacetylases, SRT1 and HDT1, and investigated their effects on flowering time under short days conditions. Two T-DNA insertion mutant *srt1* and *hdt1* showed early flowering and delayed flowering under short days respectively but showed no clear difference with wild-type plant under long days. The flowering phenotype of the mutants is linked to the changes in expression of floral genes under short days, indicating that SRT1 and HDT1 are involved in flowering control by regulating gene expression. The further studies on SRT1 and HDT1 would provide us with more clues to understand the flowering time control through deacetylases under short days.

3.2 Material and Methods

3.2.1 Plant materials and growth conditions

The *Arabidopsis* (*Arabidopsis thaliana*) Columbia-0 (Col-0) was used as the wild-type in this study. *srt1* (Sail_552_E02), *hdt1* (Salk_209141C) were used in this study. Standard growth conditions were 22°C under illumination with white fluorescent light. The photoperiodic cycle was 16 hours light/ 8 hours dark (long days) or 8 hours light/ 16 hours dark (short day). For the vernalization treatments, the surface of seeds was sterilized and spread on agar-solidified germination medium, and grown for 10 days in a 22°C growth chamber of short day condition and transferred to 4°C growth chamber for 40 days under short-day photoperiods.

3.2.2 T-DNA insertion mutant screening

T-DNA insertion mutants of SRT1 (AT5G55760) and HDT1 (AT3G44750) were ordered from the *Arabidopsis* Biological Resource Center (ABRC). Primers were designed to amplify the specific sequences upstream and downstream of the T-DNA insertion and used with T-DNA specific primers for following PCR. To amplify the T-DNA insertions of SRT1 and HDT1, LB3 (5'-TAGCATCTGAATTTCATAACCAATCTCGATACA-3') and o8474 (5'-ATAATAACGCTGCGGACATCTACATTTT-3') were used with gene-specific primers for PCR, respectively. Two PCR experiments were performed in sequence to identify individuals that were homozygous or heterozygous for the T-DNA insertion. The sizes of the T-DNA insertion bands were determined by the position of the primer in each gene. More than 3 individual lines of each mutant of *SRT1* and *HDT1* were collected and maintained for the further studies.

Gene	Name	Sequence (5' to 3')
SRT1	SRT1-LP	TTGTGGTATTCCTGATTTCCG
	SRT1-RP	AAGATCAGCCTTTTTGCAGTG
HDT1	HDT1-LP	GAAGCCTGCATCAAGCAAGAA
	HDT1-RP	GCCAACAGATTTGTGGAAACAG

Table 5. Gene-specific primers used for genotyping

3.2.3 Flowering time analysis

Seeds on plates were stratified at 4°C for two days before being transferred to a short day growth chamber at 22°C for seven to ten days. In a short day chamber, plates were exposed to 8 hours light/ 16 hours dark per day. Seedlings were then transplanted before cold exposure to be used as a non-vernalized, NV sample. Some seedlings were transferred to 4°C refrigerator (complete with a light cycle set at 8 hours light/16 hours dark per day) to be treated with vernalization. Seedlings on plates were exposed to 40 days of vernalizing cold. Additionally, plates were brought back to the 22°C short day growth chamber for 10 days after 40V. Ten to twelve seedlings of each line of each time points were transplanted to the soil to be used as a 40days-vernalized sample, 40V. Rosette leaves of the plants were counted, and an average of the number amongst replicates was used to determine the flowering phenotype under each condition and lines.

3.2.4 RNA extraction and quantitative real-time PCR analysis

Ten-day-old seedlings were harvested for RNA extraction. Total RNA was extracted using the TRIzol Reagent (Thermo Fisher Scientific). Before reverse transcription, total RNA was treated for 30 minutes at 37C with RNase-free DNases I (Invitrogen) to eliminate contaminated genomic DNA, and then total RNA (2 to 3

microgram) was used for cDNA synthesis using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR reaction was done using SYBR green dye reaction mixture (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reaction was performed on ViiA 7 Real-Time PCR system (Thermo Fisher Scientific). PCR primers used for quantitative real-time PCR analysis are listed in Table 2. The relative expression level of each gene was normalized using PP2A (AT1G13320) as described previously (Czechowski et al., 2005).

Gene	Name	Sequence (5' to 3')
FLC	FLC-real-F	GCCAAGAAGACCGAACTCATGTTGA
	FLC-real-R	CAACCGCCGATTTAAGGTGGCTA
FT	FT-real-F	GCTACAACTGGAACAACCTTTGGCA
	FT-real-R	GGCCGAGATTGTAGATCTCAGCAA
PP2A	PP2A-real-F	TATCGGATGACGATTCTTCGTGCAG
	PP2A-real-R	GCTTGGTCGACTATCGGAATGAGAG

Table 6 Primers used for RT-PCR in this study

3.3 Results

3.3.1 Characterization of T-DNA mutants

We first isolated a mutant carrying a T-DNA insertion in the middle of the *SRT1* (Sail_552_E02: T-DNA in the fifth intron) and named *srt1*. Later, we isolated more alleles of the *SRT1* gene, however, we focused on the first allele, *srt1*, for the flowering time analysis. We performed RT-PCR for the *SRT1* transcript to determine this allele is the null allele and the result displayed that *srt1* mutant did not express the *SRT1* transcript, indicating that *srt1* is a null mutant (Fig 21). We also isolated a mutant

carrying a T-DNA insertion in the third exon of the *HDT1* (Gabi_355_H03: T-DNA in the third exon) and named *hdt1*. Similar to the approach to study *srt1*, we focused on the first identified allele and the alleles identified later (*hdt1-2, hdt1-3*) were maintained for the future research. *hdt1* mutant displayed the greatly decreased level of *HDT1* transcript but still a bit detectable level in RT-PCR. However, the full transcript of *HDT1* was not detectable in *hdt1* mutant by PCR (Fig 22).

3.3.2 *srt1* mutant exhibits early flowering under short days

To investigate the function of SRT1 in the flowering pathway, we measured the number of leaves at flowering in wild-type and *srt1* mutant grown under short day condition (8h/16h light/dark) without vernalization. The *srt1* mutant generated the reduced number of leaves at flowering compared to the wild-type indicating that the *srt1* is an early flowering mutant under short days (Fig 23). The distribution of leaves numbers of the *srt1* mutant and wild-type also showed that *srt1* mutant mostly flowered before the number of leaves reached 50 whereas wild-type flowered when the number of leaves becomes more than 50 (Fig 23). We further investigated the effect of vernalization on the early flowering phenotype of the *srt1* by measuring leaves numbers of plants grown under short days after the 40 days of cold treatment when seedling stage. The *srt1* still flowered a bit earlier than wild-type, however, the early flowering phenotype was diminished in the *srt1* treated with vernalization (Fig 24). Because of this observation that vernalization corrected the early flowering of *srt1*, we tested whether *srt1* also flowers early under other conditions, such as long days, or *srt1* only displays the early flowering phenotype only under short days. We measure the number of leaves at the flowering of *srt1* and wild-type grown under long days (16h/8h light/dark) and observe that *srt1*

flowered normally similar to wild-type (Fig 25). These results indicated that *srt1* flower early specifically under short days and vernalization corrects the loss of *SRT1*.

3.3.3 Molecular characterization of *srt1* mutant

Due to the early flowering phenotype of *srt1*, we assumed that the expressions of several floral genes were affected in *srt1*. To test this, we performed RT-PCR to determine the level of *FLC* and *FT* transcript in *srt1* with wild-type control. As we expected, the level of *FLC* was decreased, and the level of *FT* was slightly increased in *srt1* (Fig 26). Previously, the repression of *FLC* expression in the different allele of the *srt1* (Salk_092181) has been reported (Bond et al., 2012) and *srt1 srt2 RNAi* mutant/knockout plants exhibited a lower level of *FLC* transcript than each single mutant suggesting SRT1 and SRT2 function redundantly on *FLC* regulation. Therefore, it is needed to generate the double mutant of *SRT1* and *SRT2* to confirm the regulation of *FLC* by these two SIR class histone deacetylases under short days for further study.

3.3.4 *hdt1* mutant exhibits late flowering under short days

In order to study the function of HDT1 in flowering control, we measured the number of leaves at flowering in wild-type and the *hdt1* mutant grown under short day condition (8h/16h light/dark) without vernalization. We observed that *hdt1* flower slightly later than wild-type plant under short day (Fig 27). The average number of leaves in *hdt1* was slightly higher than wild-type. However, the distribution of leave numbers of *hdt1* displayed that some pool of *hdt1* mutant plants showed clearly delayed flowering and some were not. This observation raised the possibility that phenotype of *hdt* could be affected by other factors such as the redundancy with other *HDT1* genes or conditional effects from the experiment.

We then tested the effect of vernalization on this slightly late flowering phenotype of the *hdt1* by measuring leaves numbers of plants grown under short day after the 40 days of cold treatment. As a result, the slightly late flowering of *hdt1* was corrected after the vernalization treatment (Fig 28). Similar to the distribution of leave number of *hdt1* under short days without vernalization, some pool of the *hdt1* with vernalization also displayed the late-flowering, and some were not. The reason for the broad distribution of flowering phenotype of the *hdt1* including the wild type like mutants and clear late-flowering mutants in one pool remains unclear. To confirm the late-flowering phenotype, the further investigation is required. We measure the number of leaves at flowering of *hdt1* and wild-type that was grown under long days (16h/8h light/dark) and observe *hdt1* flowered normally similar to wild-type (Fig 29).

3.3.5 Molecular characterization of the *hdt1* mutant

In order to characterize the late flowering phenotype of the *hdt1* under a short day, we measured the expression of two floral genes, *FLC* and *FT*, in *hdt1* and compared with those of wild-type. We performed RT-PCR to determine the level of *FLC* and *FT* transcript in the *hdt1* and wild-type control. As a result, the level of *FLC* transcript was slightly increased, and the level of *FT* was decreased in the *hdt1* (Fig 30). The changes in expression of *FLC* and *FT*, were subtle in the *hdt1*, explaining the mild flowering phenotype of a *hdt1*.

3.4 Discussion

In this study, we found two deacetylases exhibited the distinct flowering phenotypes of the mutants specifically grown under short days. The mutant of *SRT1*

exhibited the accelerated flowering, and the mutant of *HDT1* exhibited the delayed flowering (Fig 23, Fig 27). The roles of deacetylases in flowering control have not been studied thoroughly, and only a few deacetylases have been reported for their functions in flowering. HDA6 is one of the well-studied deacetylases in *Arabidopsis,* and the role of HDA6 in flowering is to repress the expression of *FLC* and other *FLC* clade genes (Yu et al., 2011). Interestingly, HDA6 repress the expression of *FLC* through the interaction with the histone demethylase, FLD, and another demethylation complex. Therefore, the loss of HDA6 in mutant caused the delayed flowering by the increased levels of histone H3 acetylation as well as H3K4 trimethylation (Yu et al., 2011). Another deacetylase, HDA5, is involved in flowering time control to regulate the expression of *FLC*. However, the HDA5 functioned through the interaction with HDA6 and played a similar role to HDA6 (Luo et al., 2015), indicating that two known deacetylases are associated with the same flowering mechanism. Since the mutants of *HDA5* and *HDA6* have a delayed flowering phenotypes under both long days and short days, the roles of two deacetylases are rather the general control of *FLC* expression than the specific control governed by the particular condition. Compared to roles HDA6 and HDA5, the roles of SRT1 and HDT1 appear more specific under short days. *srt1* and *hdt1* showed the early and late-flowering phenotypes under short days, and the mutant flowered similar to wild-type when grown under long days (Fig 23, Fig 25, Fig 27, Fig 29). These results suggest that the genes affected by the loss of *SRT1* or *HDT1* might be specific genes function only under short days for which the further studies are required.

Conventionally, the role of HDACs has been considered to be associated with inactive genes and inhibitor of active genes. However, the level of *FLC* transcripts was reduced in *srt1* (Fig 26). Thus, we assume that the role of SRT1 at *FLC* is not a conventional repressor or SRT1 might negatively regulate the unknown upstream

regulator of *FLC*. The main repressor of *FLC* in the vernalization pathway, *VIN3*, was analyzed as a target SRT1 and SRT1 in the previous study (Bond et al., 2009). However, the level of *VIN3* was not altered in *srt2* single mutant and *srt2* mutant /*SRT1*synmiRNAi plants. In addition, there was no change in expression of genes from the autonomous pathway or components of PRC2 complex in the *srt2* mutant /*SRT1*synmiRNAi plants (Bond et al., 2009), indicating that the SRT1 does not control the known *FLC* regulator in either the vernalization pathway or the autonomous pathway, rather controls unknown *FLC* regulator. Also, *srt1* plants responded to vernalization treatment. In order to search for the genes under the control of SRT1, we would consider an RNA-Seq experiment using *srt1* and analyze the differentially expressed genes in *srt1* and wild-type. We consider to investigating *srt2* for flowering time control as well as the possible redundancy of SRT2 with SRT1.

The HD-tuin type of HDACs was first discovered in maize (Lusser et al., 1997). Three genes encoding HD-tuin HDAC in maize and four genes in Arabidopsis have been found by a comparative analysis (Dangl et al., 2001). HD-tuin HDACs are present only in higher plant species, and this specificity to plants suggests the unique features and functions of proteins in this group (Grandperret et al., 2014). The study of maize HD-tuin protein revealed that it tightly bound to chromatin, and they are located mostly in the nucleolus, sharing homology with other nucleolar proteins (Lusser et al., 1997). Thus, the maize HD-tuin protein was considered to be involved in rDNA chromatin organization and expression. The Arabidopsis HD-tuin proteins, HDT1, HDT2, HDT3, and HDT4, are supposed to be involved in the repression of transcription as well. However, little is known about the mechanism of HD-tuin proteins, possible interacting partners, and downstream targets (Wu et al., 2000). It was reported that the silencing of *HDT1* resulted in aborted seeds without general pleiotropic effects (Wu et al., 2000) and ectopic

expression of *HDT1* resulted in some pleiotropic developmental defects (Zhou et al., 2004). *HDT2* and *HDT3* appeared to have identical spatial expression patterns and nucleolar localization with *HDT1* (Zhou et al., 2004) and the suppression of *HDT1* by an antisense construct also partially suppressed the expression of other HD-tuin genes (Wu et al., 2000), implying that they might carry the similar functions under the redundancy. These previous reports about HD-tuin proteins guide the future direction of our study in two ways. First, to analyze the flowering phenotypes of the higher order of mutants of *HDT1~3* genes might reveal the clear redundancy of these proteins in flowering time control. Since overexpressor of HD-tuin proteins displayed great abnormalities in development (Zhou et al., 2004), it might give us more reliable information to generate the higher order of mutant, *hdt1 hdt2 hdt3*, and to analyze the short day flowering phenotype of the triple mutant. Second, we would consider a ChIP-Seq experiment using the HDT1 transgenic plant. HDT1 is localized in the nucleus (Zhou et al., 2004) and show pleiotropic effects when it is over-expressed, indicating that HDT1 may function in multiple developmental processes. Due to the specificity of HD-tuin protein in plant species, the genome-wide association of HDT1 might reveal the clues for a novel plant specific-mechanism.

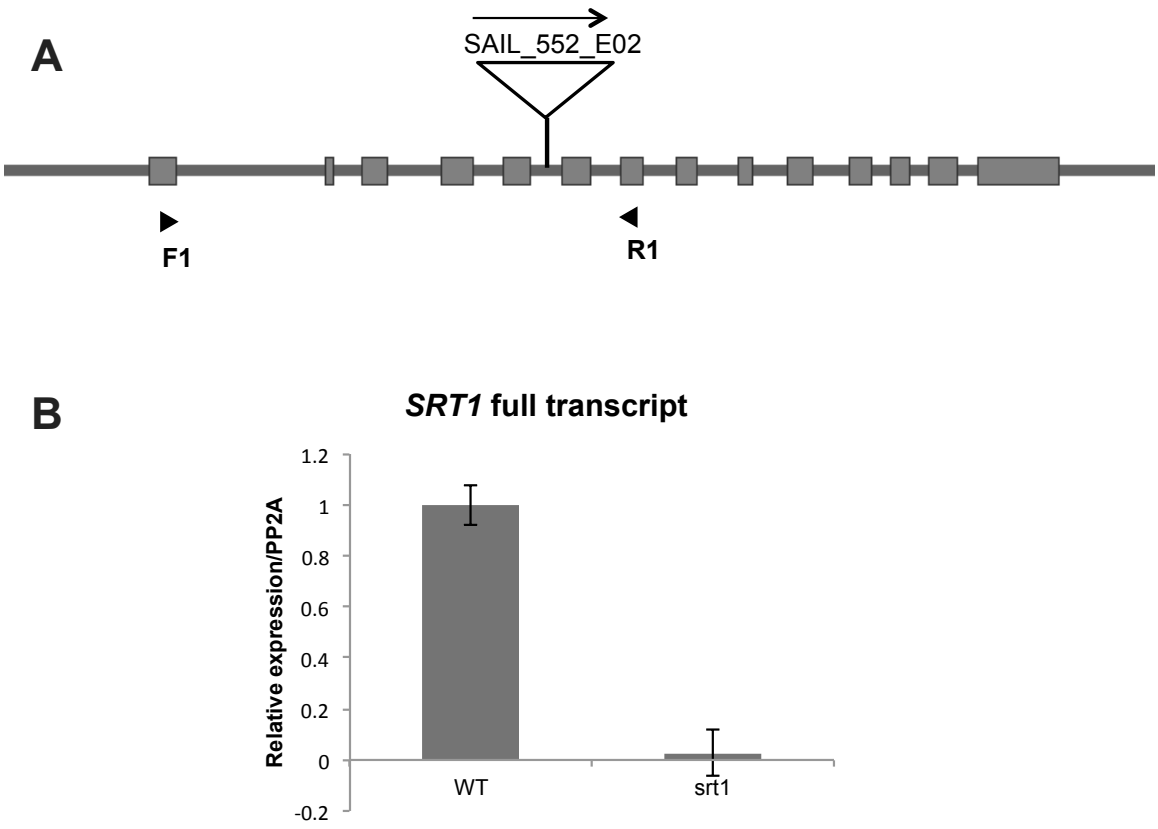

Figure 21. Characterization of *srt1* mutant

(A) A schematic representation of the genomic structure of *SRT1* and positions of the T-DNA insertion and primers used to detect the full transcript of *SRT1* are indicated (primer: F1 and R1). (B) The levels of *SRT1* full transcripts in WT and the *srt1* mutant.

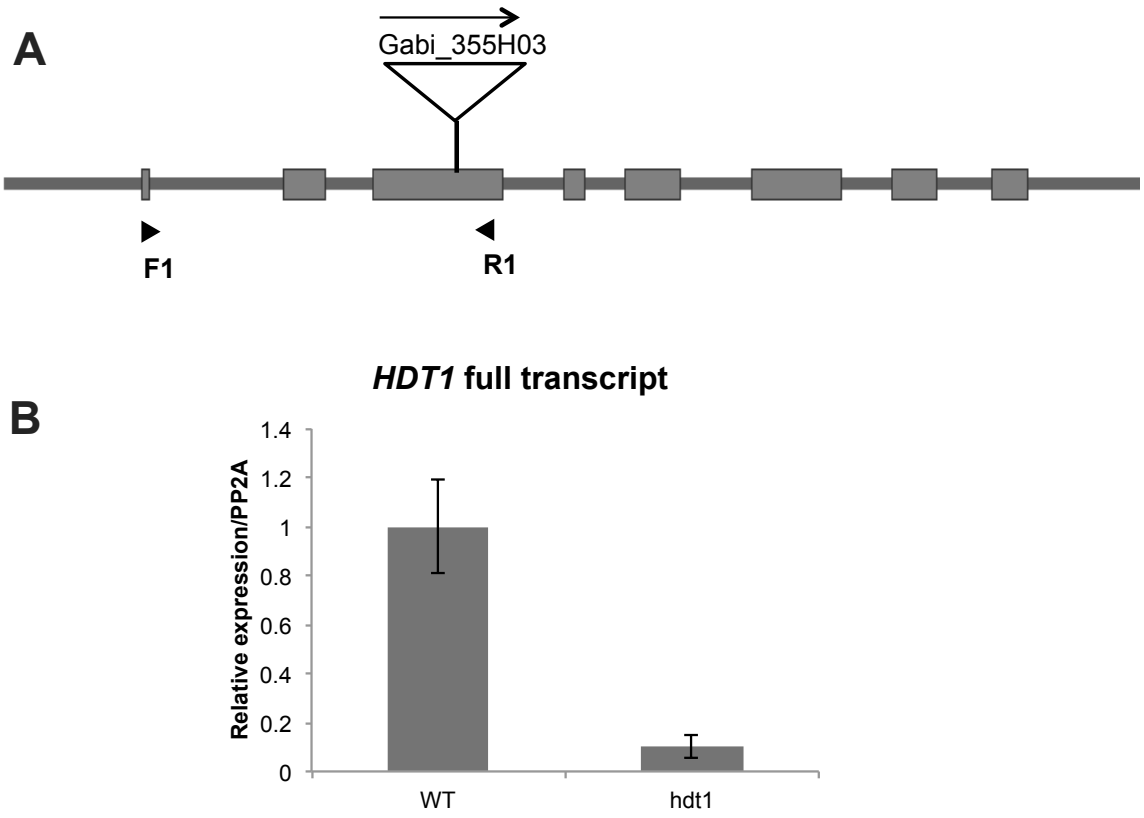

Figure 22. Characterization of *hdt1* mutant

(A) A schematic representation of the genomic structure of *HDT1* and positions of the T-DNA insertion and primers used to detect the full transcript of *HDT1* are indicated (primer: F1 and R1). (B) The levels of *HDT1* full transcripts in WT and the *hdt1* mutant.

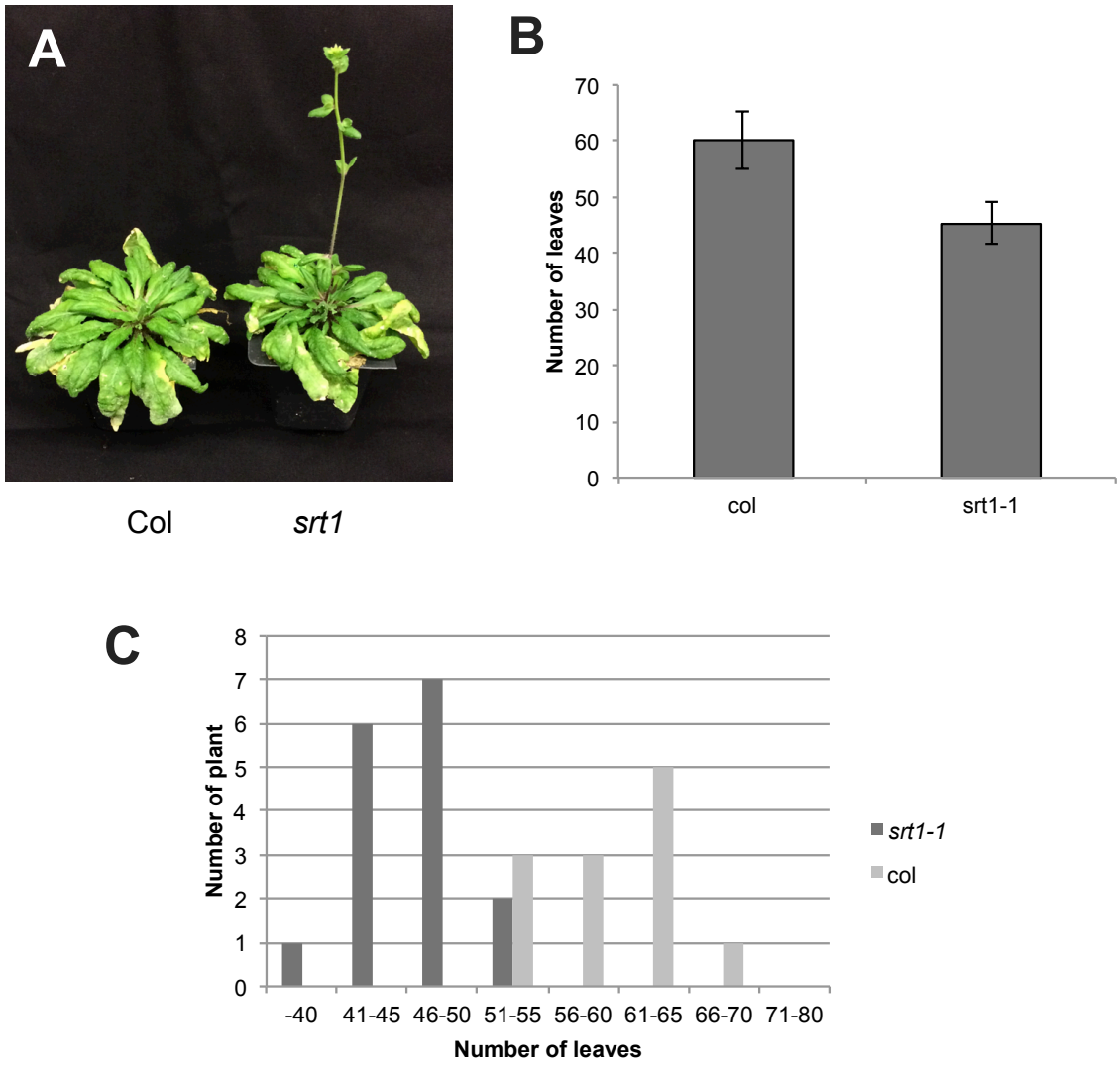

Figure 23. Flowering phenotype of *srt1* mutant under short days without vernalization

(A) The flowering of wild-type (Col) and the *srt1* mutant under short days (B) An average numbers of leaves at the time of flower in wild-type and the *srt1* mutant. (C) The distribution of the leave numbers in wild-type and the *srt1* mutant under a short day.

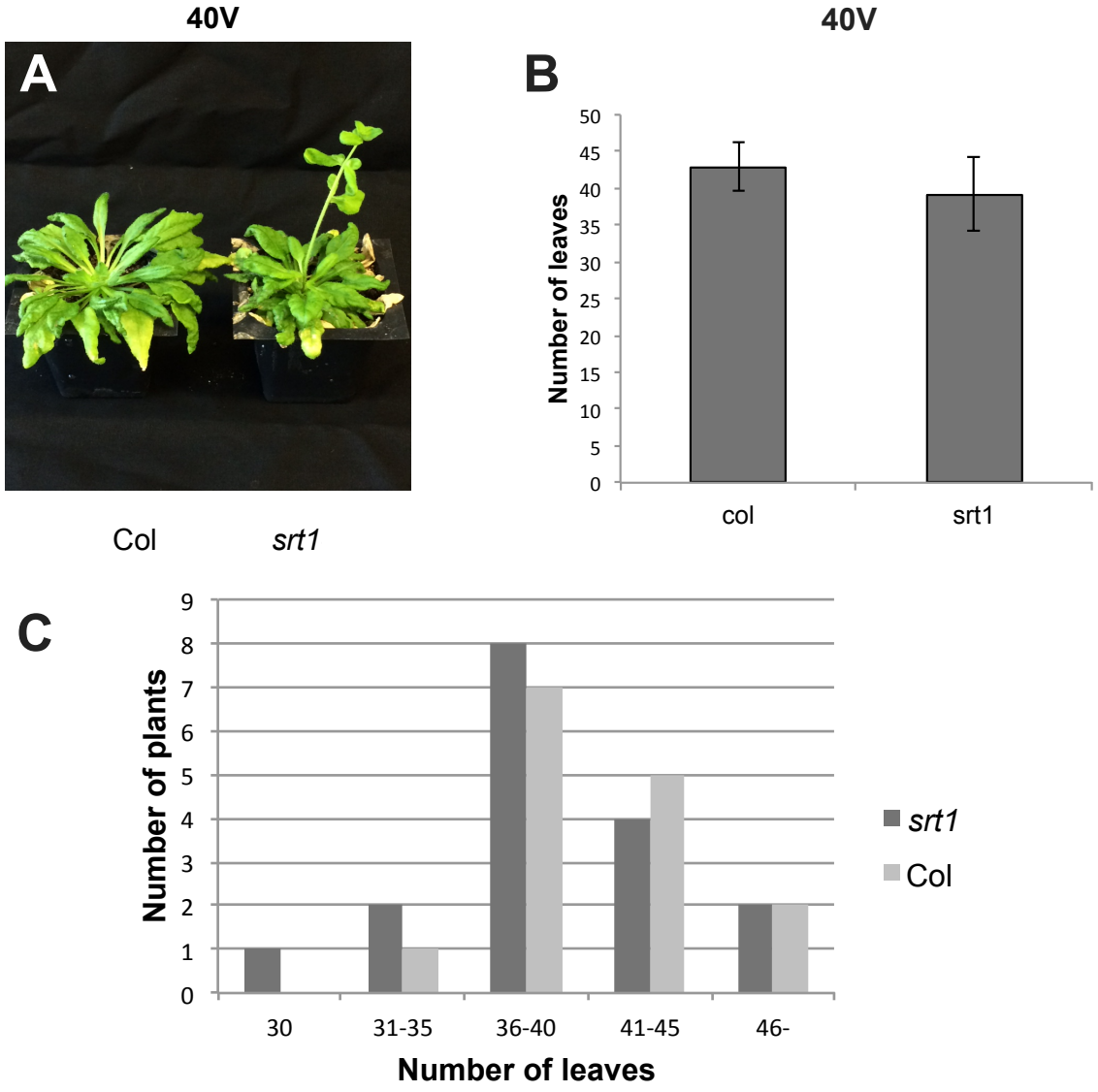

Figure 24. Flowering phenotype of *srt1* mutant with vernalization treatment

(A) The flowering of wild-type (Col) and the *srt1* mutant with 40 days of vernalization treatment grown under short days (B) An average numbers of leaves at the time of flower in wild-type and *srt1* mutant. (C) The distribution of the leave numbers in wild-type and the *srt1* mutant with vernalization under short days.

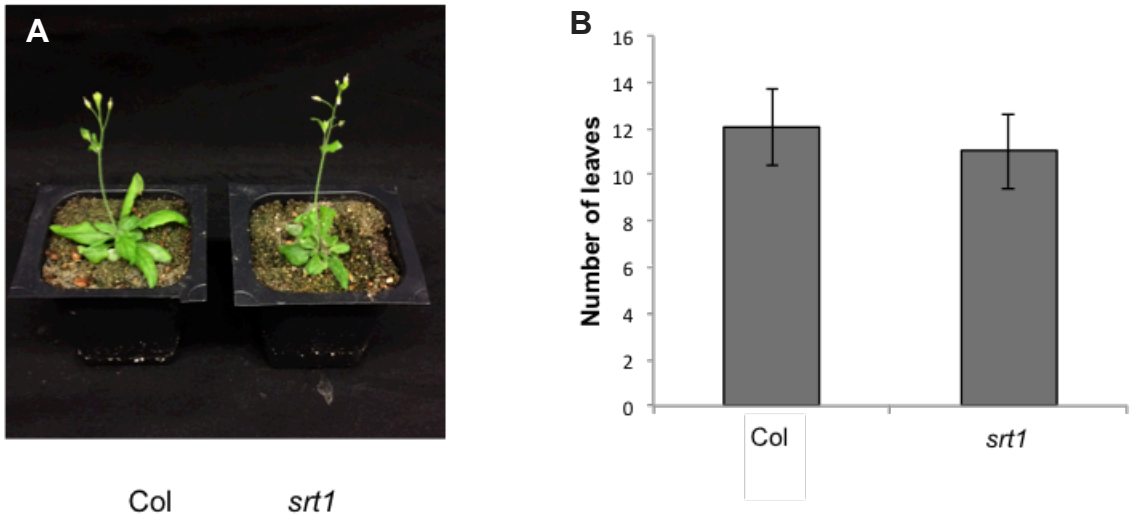

Figure 25. Flowering phenotype of the *srt1* mutant under long days

(A) The flowering of wild-type (Col) and the *srt1* mutant under long days (B) An average number of leaves at the time of flower in wild-type and the *srt1* mutant.

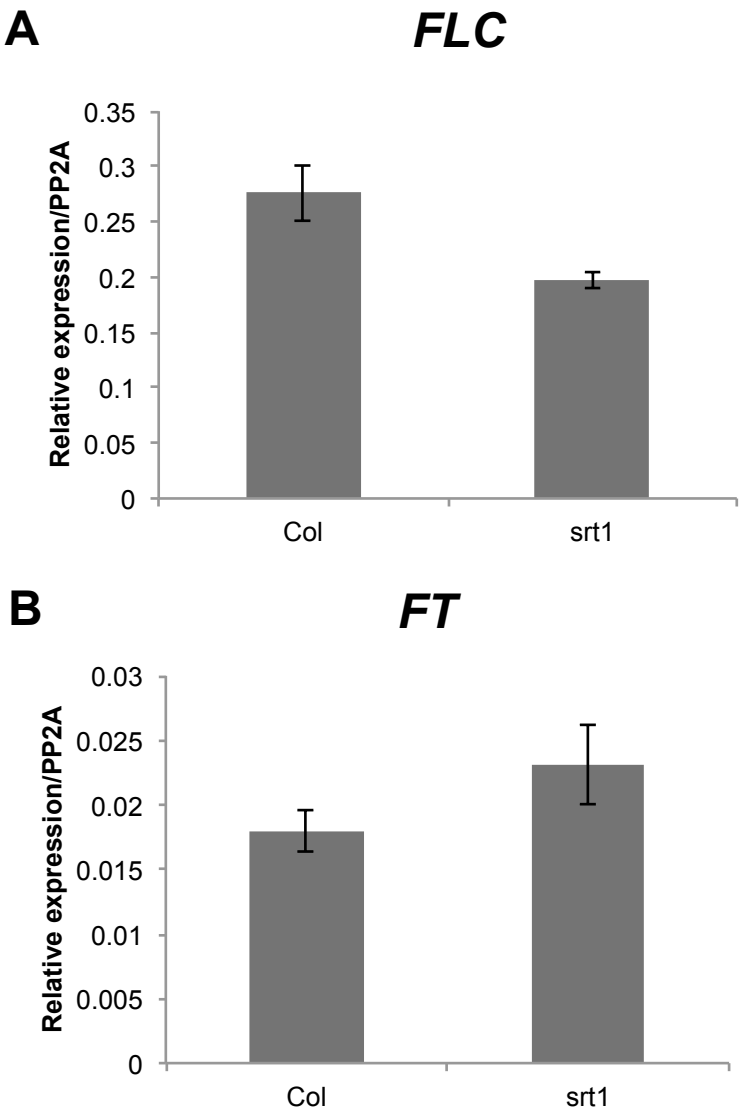

Figure 26. Repression of *FLC* and *FT* in the *srt1* mutant

(A) Relative expression of *FLC* in wild-type and the *srt1* mutant (B) Relative expression of *FT* in wild-type and the *srt1* mutant

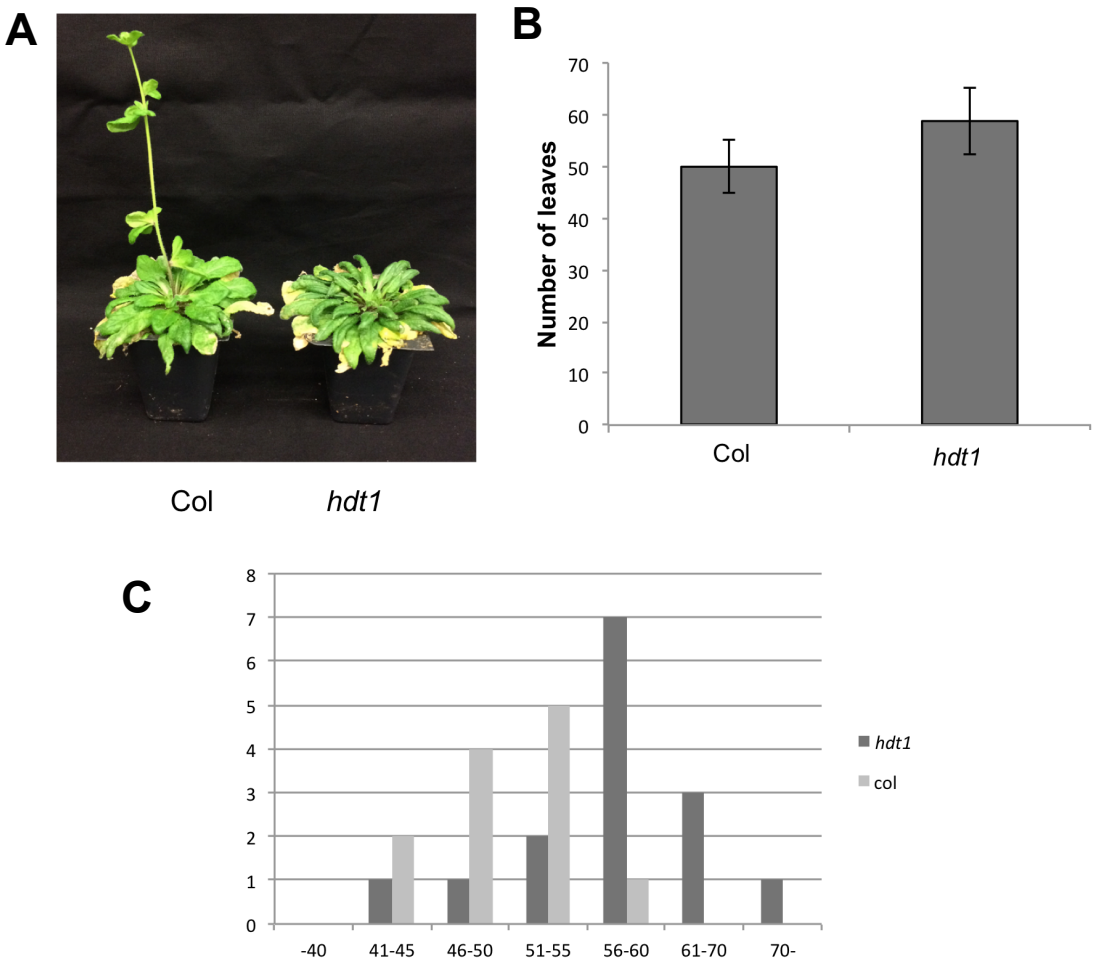

Figure 27. Flowering phenotype of the *hdt1* mutant under short days without vernalization

(A) The flowering of wild-type (Col) and the *hdt1* mutant under short days (B) An average number of leaves at the time of flower in wild-type and *hdt1* mutant. (C) The distribution of the leave numbers in wild-type and the *hdt1* mutant under short days.

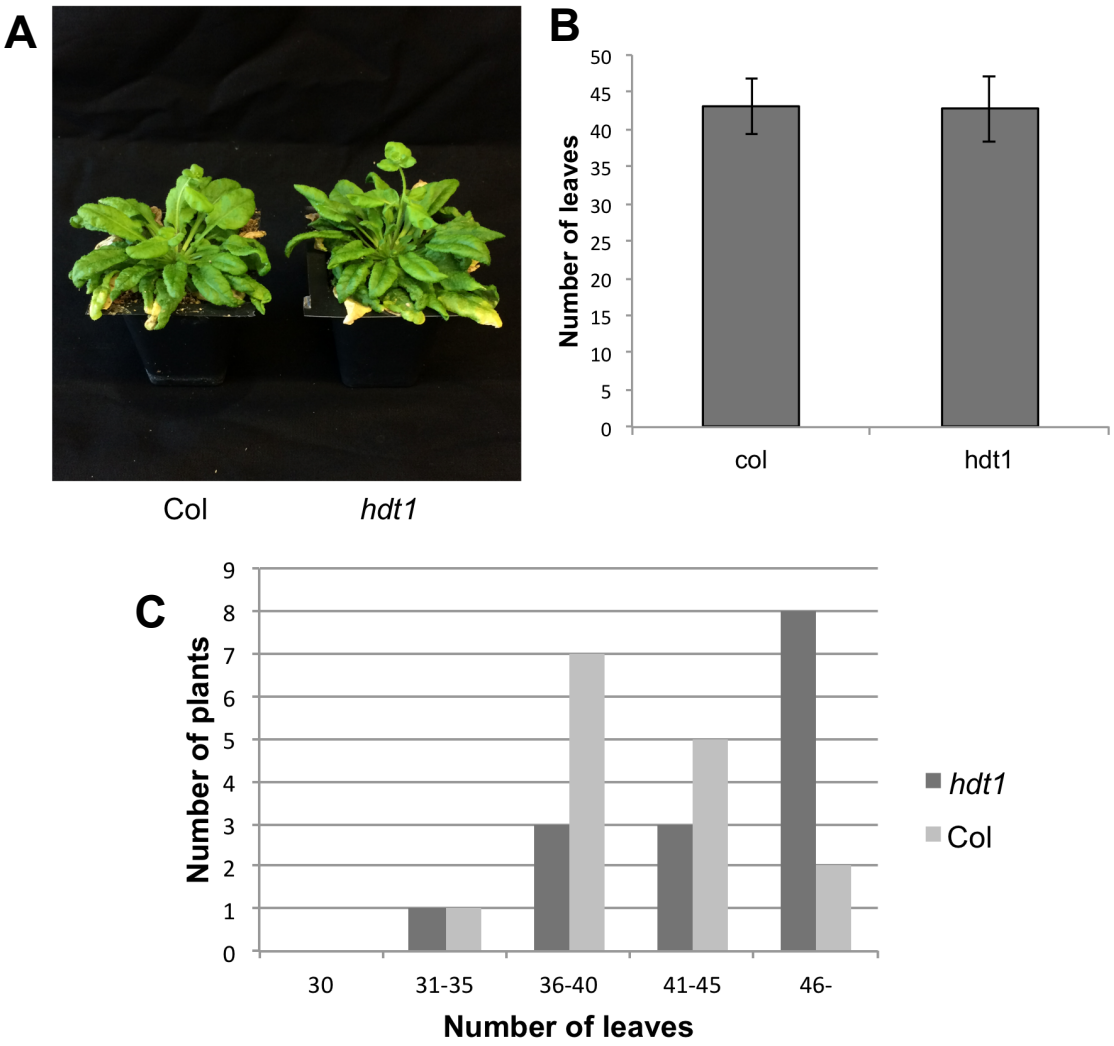

Figure 28. Flowering phenotypes of the *hdt1* mutant under short days without vernalization

(A) The flowering of wild-type (Col) and the *hdt1* mutant under short days (B) An average number of leaves at the time of flower in wild-type and the *hdt1* mutant. (C) The distribution of the leave numbers in wild-type and the *hdt1* mutant under short days.

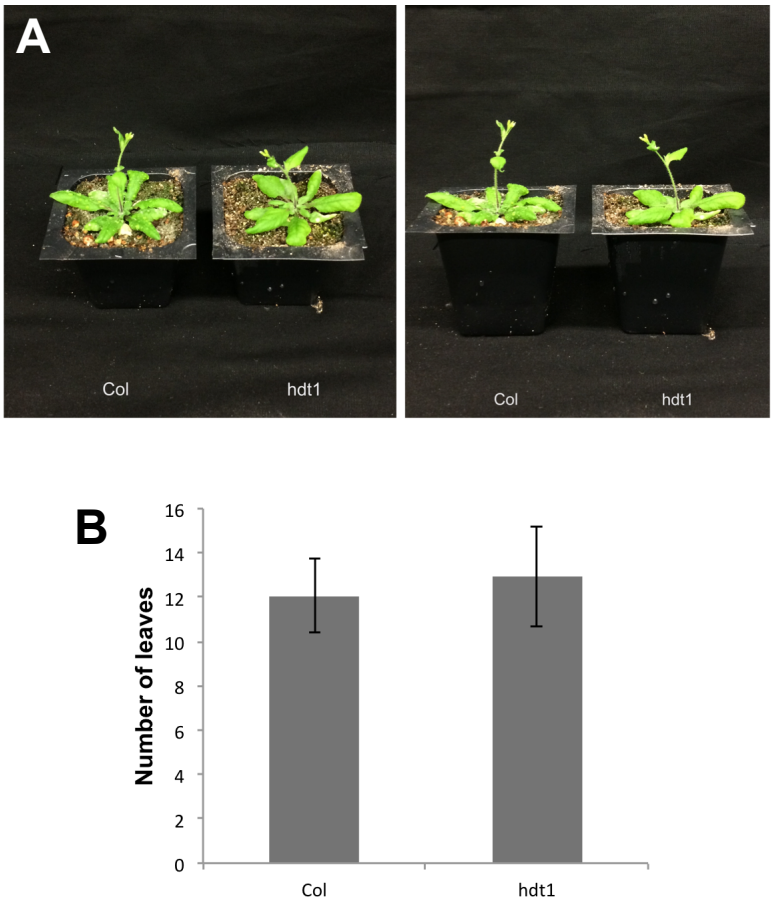

Figure 29. Flowering phenotypes of *hdt1* mutant under long day

(A) The flowering of wild-type (Col) and the *hdt1* mutant under long days (B) An average number of leaves at the time of flower in wild-type and the *hdt1* mutant.

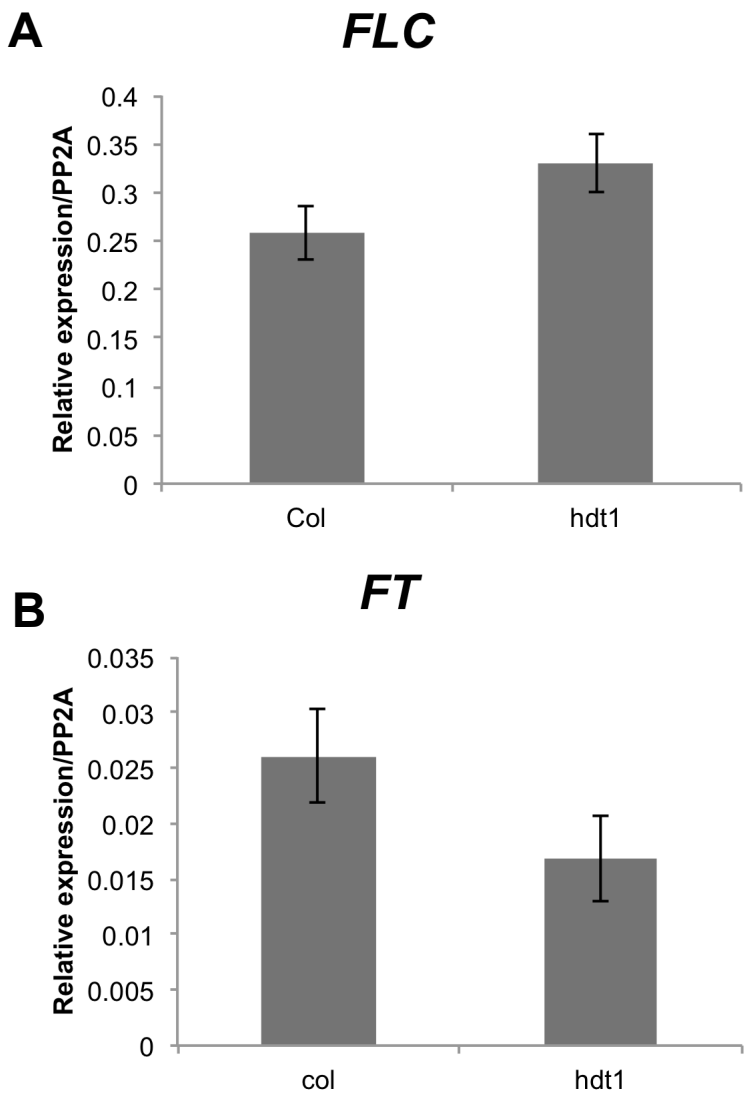

Figure 30. Expression of *FLC* and *FT* in the *hdt1* mutant

(A) Relative expression of *FLC* in wild-type and the *hdt1* mutant (B) Relative expression of *FT* in wild-type and the *hdt1* mutant

<Bibliography/References>

Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K 2007. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature

Alvarez-Venegas R, Avramova Z 2001. Two Arabidopsis homologs of the animal trithorax genes: a new structural domain is a signature feature of the trithorax gene family. Gene

Aufsatz W, Mette MF, van der Winden J, Matzke M, Matzke AJM 2002. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. EMBO J

Bastow R, Joshua S, Mylne J, Lister C, Lippman Z, Martienssen RA, Dean C 2004. Vernalization requires epigenetic silencing of FLC by histone methylation. Nature

Balasubramanian S, Sureshkumar S, Lempe J, Weigel D 2006. Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. Plos Genetics.

Baumbushch LO, Thorstensen T, Krauss V, Fischer A, Naumann K, Assalkhou R, Schulz I, Reuter G, Aalen RB 2001. The Arabidopsis thaliana genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. Nucleic Acids Research

Baurle I, Smith L, Baulcombe DC, Dean C 2007. Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. Science.

Berger SL 2007. The complex language of chromatin regulation during transcription. Nature

Bernier G, Kinet JM, Sachs RM 1981. The Physiology of flowering. CRC Press, Boca Raton.

Berr A, Menard R, Heitz T, Shen WH 2012. Chromatin modification and remodeling: a regulatory landscape for the control of Arabidopsis defenses upon pathogen attack. Cellular Microbiology

Bohelenius H, Huang T, Charbonnel-Campass L, Brunner AM, Jansson S, Strauss SH, Nilsson O 2006. CO/FR regulatory module controls timing of flowering and seasonal growth cessation in trees. Science

Bond DM, Dennis ES, Pogson BJ, Finnegan EJ 2009. Histone Acetylation, *VERNALIZATION INSENSITVE 3*, *FLOWERING LOCUS C*, and vernalzation response. Mol Plant

Bordoli L, Netsch M, Luthi U, Lutz W, Eckner R 2001. Plant orthologs of p300/CBP: conservation of a core domain in metazoan p300/CBP acetyltransferase-related proteins. Nucleic Acids Res

Boycheva I, Vassileva V, Iantcheva A 2014. Histone acetyltransferases in plant development and plasticity. Current Genomics

Cao R, Wang L. Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y 2002. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science

Cao Z, Li Y, Chen Z, Wang H, Zhang M, Zhou N, Wu R, Ling Y, Fang F, Zhang Y 2015. Genome-wide dynamic profiling of histone methylation during nuclear transfer-mediated porcine somatic cell reprogramming. PLoS ONE

Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, Goodrich J 2004. Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development

Chen Y, Yamaguchi Y, Tsugeno Y, Yamamoto J, Yamada T, Nakamura M, Hisatake K, Handa H 2009. DSIF, the Paf1 complex, and Tat-SF1 have nonredundant, cooperative roles in RNA polymerase II elongation. Genes Dev.

Chi P, Allis CD, Wang GG 2010. Covalent histone modification—miswritten, misinterpreted and mis-erased in human cancers. Nature Reviews Cancer

Choi K, Kim J, Hwang HJ, Kim S, Park C, Kim SY, Lee I 2011. The FRIGIDA Complex activates transcription of *FLC*, a strong flowering repressor in *Arabidopsis*, by recruiting chromatin modification factors. Plant Cell

Chouard P 1960. Vernalization and its relations to dormancy. Annual Review of Plant Physiology

Chen ZJ, Pikarrad CS 1997. Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleoar domainance. Genes Dev

Chua YL, Watson LA, Gray JC 2003. The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. Plant Cell

Cigliano RA, Cremona G, Paparo R, Termolino P, Perrella G, Gutzat R, Consiglio MF, Conicella C 2013. Histone deacetylase AtHDA7 is required for female gametophyte and embryo development in *Arabidopsis*. Plant Physiol

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH, Helin K 2006. The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. Nature

Cohen R, Schocken J, Jaldis A, Vlachonasios KE, Hark AT, McCain ER 2009. The histone acetyltransferase GCN5 affects the inflorescence meristem and stamen development in Arabidopsis. Planta

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakounits A, Farrona S, Gissot L, Turnbull C, Coupland G 2007. FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science

Davis S 2009. Integrating hormones into the floral-transition pathway of Arabidopsis thaliana. Plant, Cell and Environment.

De Lucia F, Crevillen P, Jones AM, Greb T, Dean C 2008. A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of FLC during vernalization. PNAS

Deng W, Liu C, Pei Y, Deng X, Niu L, Cao X. 2007. Involvement of the histone acetyltransferase AtHAC1 in the regu- lation of flowering time via repression of FLOWERING LOCUS C in Arabidopsis. Plant Physiol.

Dangl M, Brosch G, Haas H, Loidl P, Lusser A 2001. Comparative analysis of HD2 type histone deacetylases in higher plants. Planta

Dennis ES, Peacock WJ 2007. Epigenetic regulation of flowering. Current Opinion in Plant Biolgody

Dou Y, Milne TA, Ruthenburg AJ, Lee S, Lee JW, Verdine GL, Allis CD, Roeder RG 2006. Regulation of MLL1 H3K4 methyltransferase activity by its core components. Nat Struct Mol Biol.

Earley KW, Shook MS, Brower-Toland B, Hicks L, Pikaard CS 2007. In vitro specificities of Arabidopsis co-activator histone acetyltransferases: implications for histone hyperacetylation in gene activation. Plant J

Finnegan EJ, Dennis ES 2007. Vernalization-induced trimethylation of histone H3 lysine 27 at FLC is not maintained in mitotically quiescent cells. Curr Biol

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J 1999. GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO Journal

Frye RA 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun.

Fuchs J, Demidov D, Houben A, Schubert I 2006, Chromosomal histone modification patterns from conservation to diversity. Trends Plant Sci

Gaudin V 2001. Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis. Development

Greb T, Mylne JS, Crevillen P, Geraldo N, An H, Gendall AR, Dean C 2007. The PHD finger protein VRN5 functions in the epigenetic silencing of Arabidopsis FLC. Current Biology

Greer EL, Shi Y 2012. Histone methylation: a dynamic mark in health, disease and inheritance. Nature Review Genetics

Gendall AR, Levy YY, Willson A, Dean C 2001. The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell

Geraldo N, Baurle I, Kidou SI, Hu X, Dean C 2009. FRIGIDA delays flowering in Arabidopsis via a cotranscriptional mechanism involving direct interaction with the nuclear cap-binding complex. Plant Physiology

Gonzalez D, Bowen AJ, Carroll TS, Conlan RS 2007. The transcription corepressor LEUNIG interacts with the histone deacetylase HDA19 and mediataor components MED14 (SWP) and CDK8 (HEN3) to repress transcription. Mol Cell.Biol.

Grandperret V, Nicolas-Francès V, Wendehenne D, and Bourque S 2014. Type-II histone deacetylases: elusive plant nuclear signal transducers. Plant Cell Environ.

Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL 2001. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J. Biol. Chem.

Han SK, Song JD, Noh YS, Noh BS 2007. Role of plant CBP/ p300-like genes in the regulation of flowering time. Plant J.

Han Z, Yu H, Zhao Z, Hunter D, Luo X, Duan J, Tian L 2016. AtHD2D Gene Plays a Role in Plant Growth, Development, and Response to Abiotic Stresses in Arabidopsis thaliana Front. Plant Sci

He HH, Meyer CA, Shin H, Bailey S, Wei G, Wang Q, Zhany Y, Xu K, Ni M, Lupien M 2010. Positioned nucleosomes flanking a labile nucleosome characterize transcriptional enhancers. Nature Genetics

Hebbes TR, Thorne AW, Crane-Robinson C 1998. A direct link between core histone acetylation and transcriptionally active chromatin. EMBO

He Y, Doyle MR, Amasino RM 2004. PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev

He Y, Amasino RM 2005. Role of chromatin modification in flowering-time control. Trends Plant Sci

Helliwell C, Wood C, Robertson M, Peacock WJ, Dennis E 2996. The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant Journal.

Hsieh TF, Hakim O, Ohad N, Fischer RL 2003. From flour to flower: How Polycomb group proteins influence multiple aspects of plant development. Trends Plant Sci.

Hu Z, Song N, Zheng M, Liu X, Liu Z, Xing J, Ma J, Guo W, Yao Y, Peng H, Xin M, Zhou DX, Ni Z, Sun Q 2015. Histone acetyltransferase GCN5 is essential for heat

stress-responsive gene activation and thermotolerance in Arabidopsis. Plant Journal.

Huang T, Böhlenius H, Eriksson S, Parcy F, Nilsson O 2005. The mRNA of Arabidopsis gene FT moves from leaf to shoot apex and induces flowering. Science.

Jacob Y, Feng S, LeBlanc CA, Bernatavichute YV, Stroud H, Cokus S, Johnson LM, Pellegrini M, Jacobsen SE, Michaels SD 2009. ATXR5 and ATXR6 are H3K27 monomethyltranferases required for chromatin structre and gene silencing. Nat.Struct.Mol.Biol

Jeong JH, Song HR, Ko JH, Jeong YM, Kwon YE, Seol JH, Amasino RM, Noh B, Noh YS 2009. Repression of FLOWERING LOCUS T chromatin by functionally redundant histone H3 lysine 4 demethylases in Arabidopsis. PLos ONE 2009

Jiang D, Yang W, He Y, Amasino RM 2007. Arabidopsis relatives of the human lysine-specific demethylase1 repress the expression of *FWA* and *FLOWERING LOCUS C* and thus promote the floral transition. Plant Cell

Jiang D, Gu X, He Y 2009. Establishment of the winter-annual growth habit via *FRIGIDA*-mediated histone methylation at *FLOWERING LOCUS C* in Arabidopsis. Plant Cell.

Jung M, Hoffmann K, Brosch G, Loidl P 1997. Analogues of trichostatin A and trapoxin B as histone deacetylase inhibitors. Bioor. Med. Chemm. Lett.

Kang MJ, Jin HS, Noh YS, Noh B 2015. Repression of flowering under a noninductive photoperiod by the HDA9-AGL19-FT module in Arabidopsis. New Phytologist.

Katsani KR, Arredondo JJ, Kal AJ, Verrijzer CP 2001. A homeotic mutation in the trithorax SET domain impeds histone binding. Genes and Development

Ketel CS, Andersen EF, Vargas ML, Suh J, Strome S, Simon JA 2005. Subunit contributions to histone methyltransferase activities of fly and worm polycomb group complexes. Mol Cell Biol.

Kim DH, Doyle M, Sung S, Amasion R 2009. Vernalization: Winter and the timing of flowering in plants. Annual Review of Cell land Developmental Biology.

Kim J, Guermah M, Roeder RG 2010. The human PAF1 complex acts in chromatin transcription

Kim W, Latrasse D, Servet C, Zhou DX 2013. Arabidopsis histone deacetylase HDA9 regulates flowering time through repression of AGL19. Biochemical and Biophysical Research Communications

Klose RJ, Kallin EM, Zhang Y 2006. JmjC-domiain-containing proteins and histone demethylation. Nat.Rev.Genet

Klose RJ, Yamane K, Bae Y, Zhang D, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y 2006. The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature

Klose RJ, Zhang Y 2007. Regulation of histone methylation by demethylimination and demethylation. Nature Reviews Molecular Cell Biology

Ko JH, Mitina I, Tamada Y, Hyun Y, Choi Y, Amasion RM, Noh B, Noh YS 2010. Growth habit determination by the balance of histone methylation activities in Arabidopsis. EMBO

König AC, Hartl M, Pham PA, Laxa M, Boersema PJ, Orwat A, Kalitventseva I, Plöchinger M, Braun HP, Leister D, Mann M, Wachter A, Fernie AR, Finkemeier I 2014. The Arabidopsis class II sirtuin is a lysine deacetylase and interacts with mitochondrial energy metabolism. Plant Physiol

Kotake T, Takada S, Nakahigashi K, Ohto M, Goto K 2003. Arabidosis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes. Plant Cell Physiol.

Krogan NJ, Keogh MC, Datta N, Sawa C, Ryan OW, Ding H, Haw RA, Pootoolal J, Tong A, Canadien V, Richards DP, Wu X, Emili A, Hughes R, Buratowski S, Greenblatt JF 2003. A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol. Cell

Kurdistani SK, Grunstein M, 2003. *In vivo* protein-protein DNA crosslinking for genome-wide binding microarray. Methods

Lang A 1965. Physiology of flower initiation. In Encyclopedia of Plant Physiology.

Lee J, Lee I 2010. Regulation and function of SOC1, a flowering pathway integrator. Journal of Experimental Botany.

Lee K, Park OS, Jung SJ, Seo PJ 2016. Histone deacetylation-mediated cellular dedifferentiation in Arabidopsis. J Plant Physiol

Levine SS, King I, Kingston R 2004. Division of labor in Polycomb group repression. Trends in Biochemical Sciences

Liu F, Quesada V, Crevillen P, Baurle I, Swiezewski S, Dean C 2007. The Arabidopsis RNA-binding protein FCA requires a lysine-specific demthylases 1 homolog to downregulate FLC. Molecular Cell

Liu F, Marquardt S, Lister C, Swiezewski S, Dean C 2010. Targeted 3' processing of antisense transcripts triggers *Arabidopsis FLC* chromatin silencing. Science

Liu C, Li LC, Chen WQ, Chen X, Xu ZH, Bai SN 2013. HDA18 affects cell fate in Arabidopsis Root epidermis via histone acetylation at four kinase genes. Plant Cell

Liu X, Yang S, Zhao M, Luo M, Yu CW, Chen CY, Tai R, Wu K 2014. Transcriptional repression by histone deacetylases in plants. Mol Plant

Long JA, Ohno C, Smith ZR, Meyerowitz EM 2006. TOPLESS regulates apical embryonic fate in Arabidopsis. Science

Luo M, Wang YY, Liu X, Yang S, Lu Q, Cui Y, Wu K 2012. HD2C interacts with HDA6 and is involved in ABA and salt stress response in Arabidopsis. J Exp Bot

Luo M, Tai R, Yu CW, Yang S, Chen C, Lin WD 2015. Regulation of flowering time by the histone deacetylase HDA5 in *Arabidopsis*. Plant J.

Lusser A, Brosch G, Loidl A, Haas H, Loidl P 1997. Identification of maize histone deacetylase HD2 as an acidic nucleolar phosphoprotein. Science

Maison C, Almouzni G 2004. HP1 and the dynamics of heterochromatin maintenance. Nat. Rev. Mol. Cell Biol.

Makarevich G, Leroy O, Akinci U, Schubert D, Clarenz O, Goodrich J, Grossniklaus U, Kohler C 2006. Different Polycomb group complexes regulate common target genes in Arabidopsis. EMBO Rep.

Mao Y, Pavangadkar KA, Thomashow MF, Triezenberg SJ 2006. Physical and functional interactions of Arabidopsis ADA2 transcriptional coactivator proteins with the acetyltransferases GCN5 and with the cold-induced transcription factor CBF1. Biochim Biophys Acta

Mbogning J, Nagy S, Page V, Schwer B, Shuman S, Fisher RP, Tanny JC 2013. The PAF complex and Prf1/Rtf1 delineate distinct Cdk9-dependent pathways regulating transcription elongation in fission yeast. PLoS Genet.

Michaels SD, Amasino RM 1999. The gibberellic acid biosynthesis mutant ga1-3 of Arabidopsis thaliana is responsive to vernalization. Developmental Genetics.

Miller T, Krogan NJ, Dover J, Erdjument-Bromage H, Tempst P, Johnston M, Greenblatt JF, Shilatifard A. COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc.Natl.Acad.Sci.

Muller J, Hart CM, Francis M, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA 2002. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell

Mutasa-Gottgens E, Hedden P 2009. Gibberellin as a factor in floral regulatory networks. Journal of Experimental Botany

Mutskov V, Gerber D, Angelov D, Ausio J, Workman J, Dimitrov S 1998. Persistent interactions of core histone tails with nucleosomal DNA following acetylation and transcription factor binding. Mol Cell.Biol

Mylne JS, Brarrett L, Tessadori F, Mesnage S, Johnson L. Bernatavichute Y, Jacobse SE, Fransz R, Dean C 2006. LHP1, the Arabidopsis homologue of HETEROCHOROMATIN PROTEIN1, is required for epigenetic silencing of FLC. Proc Natl Acad Sci

Mylne J, Greb T, Lister C, Dean C. 2004. Epigenetic regulation in the control of flowering. Cold Spring Harbor Symposia on Quantitative Biology.

Noh YS, Bizzell CM, Noh B, Schomburg FM, Amasino RM 2004. EARLY FLOWERING 5 acts as a floral repressor in Arabidopsis. Plant Journal.

Noh YS, Amasino RM 2003, PIE, an ISWI family gene, is required for FLC activation and floral repression in Arabidopsis. Plant Cell.

Pandey R, Muller A, Napoli CA, Selinger DA, Pikaard CS, Richards EJ 2002. Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res.

Pavangadkar K, Thomashow MF, Triezenberg SJ 2010. Histone dynamics and roles of histone acetyltransferases during cold-induced gene regulation in Arabidopsis. Plant Mol Biol.

Pien S, Felury D, Mylne JS, Crevillen P, Inze D, Avramova Z, Dean C, Grossniklaus U 2008. ARABIDOPSIS TRITHORAX1 dynamically regulates FLOWERING LOCUS C activation via histone 3 lysine 4 trimethylation. Plant Cell

Puig OM, Belle's E, Lopez-Rodas G, Sendra R, Tordera V 1998. Interaction between N-terminal domain of H4 and DNA is regulated by the acetylation degree. Biochim.Biophys.Acta

Putterill J, Robson F, Lee K, Simon R, Coupland G 1995. The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell.

Rao B, Shigata Y, Strahl BD, Lief JD 2005. Dimethylation of histone H3 at lysine 36 demarcates regulatory and nonregulatory chromatin genome-wide. Molecular cEll Biology

Ratcliffe OJ, Bouveret R, Leroy O, Borghi L, Köhler C, Gruissem W, Hennig L 2001. Regulation of flowering in Arabidopsis by an FLC homolog. Plant Physiol.

Ratcliffe OJ, Kumimoto RW, Wong BJ, Riechmann JL 2003. Analysis of the Arabidopsis MADS AFFECTING FLOWERING gene family: MAF2 prevents vernalization by short periods of cold. Plant Cell

Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T 2000. Regulation of chromatin structure by site-specific histone H3 methylatransferases. Nature

Ringrose L, Paro R 2004. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annual Rev Genet.

Roudier F, Ahmed I, Colot V 2011. Integrative epigenomic mapping defines four main chromatin states in Arabidopsis. EMBO

Ruthenburg AJ, Li H, Patel DJ, Allis CD 2007. Multivalent engagement of chromatin modifications by linked binding modules. Nat.Rev.Mol Cell Biol.

Ryu H, Cho H, Bae W, Hwang I 2014. Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. Nat Communication

Saleh A, Alvarez-Venegas R, Avramova Z 2008. Dynamic and stable histone H3 methylation patterns at the Arabidopsis FLC and AP1 loci. Gene

Schmitz RJ, Sung S, Amasino RM 2008. Histone arginine methylation is required for vernalization-induced epigenetic silencing of FLC in winter-annual Arabidopsis thaliana. Proc Natl Acdad Sci.

Schönrock N, Bouveret R, Leroy O, Borghi L, Köhler C, Gruissem W, Hennig L 2006. Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway. Genes Dev

Schwartz YB, Pirrotta V 2007. Polycomb silencing mechanisms and the management of genomic programmes. Nat.Rev. Genet.

Searle I, He Y, Turck F, Vincent C, Fornara F, Krober S, Amasino R, Coupland G 2006. The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes and Development.

Servet C, Conde e Silava N, Zhou DX 2010. Histone acetyltransferase AtGCN/HAG1 is a versatile regulator of developmental and inducible gene expression in Arabidosis. Mol. Plant

Shafiq S, Berr A, Shen WH 2014. Combinatorial functions of diverse histone methylations in Arabidopsis thaliana flowering time regulation. New Phytol

Sheldon C, Burn J, Perez P, Metzger J, Edwards J, Peacock W, Dennis E 1999. The FLF MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell

Shilatifard A 2008. Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. Curr.Opin.Cell Biol.

Song YH, Lee I, Lee SY, Imaizumi T, Hong JC 2012. CONSTANS and ASYMMETRIC LEAVES 1 complex is involved in the induction of FLOWERING LOCUS T in photoperiodic flowering in Arabidopsis. Plant Journal.

Spinger NM, Napoli CA, Selinger DA, Pandey R, Cone KC, Chandler VL, Kaeppler HF, Kaeppler SM 2003. Compartive analysis of SET domain proteins in maize and Arabidopsis reveals multiple duplications preceding the divergence of monocots and dicots. Plant Physiology

Sridha S, Wu K 2006. Identification of AtHD2C as a novel regulator of abscisic acid responses in Arabidopsis. Plant J

Srikanth A, Schimd M 2011. Regulation of flowering time: all roads lead to Rome. Cellular and Molecular Life Sciences

Sterner DE, Berger SL 2000. Acetylation of histones and transcription-related factors. Microbiol Mol Bio Rev

Sung S and Amasino RM 2004. Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature

Sung S and Amasino RM 2005. Remembering winter: toward a molecular understanding of vernalization. Annual Review of Plant Biology.

Sung S, He Y, Eshoo TW, Tamada Y, Johnson L, Nakahigashi K, Goto K, Jacobsen SE, Amasino RM 2006. Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN1. Nature

Sung S, Schmitz RJ, Amasino RM 2006. A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes and Development

Tamada Y, Yun JY, Woo SC, Amasion RM 2009. ARABIDOPSIS THITHORAX-RELATED7 is required for methylation of lysine 4 histone H3 and for transcriptional activation of FLOWERING LOCUS C. Plant Cell

Tang X, Lim MH, Pelletier J, Tang M, Nguyen V, Keller WA, Tsang EW, Wang A, Rothstein SJ, Harada JJ, Cui Y 2012. Synergistic repression of embryonic programmed by SET DOMAIN GROUP8 and EMBRYONIC FLOWER 2 in Arabidopsis seeling. J.Exp.Bot

Utley RT, Ikeda K, Grant PZ, Cote J, Steger DJ, Eberharter A 1998. Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. Nature

Vakoc CR, Sachdeva MM, Wang H, Blobel GA 2006. Profile of histone lysine methylation across transcribed mammalian chromatin. Mol Cell Biol

Vlachonasios KE 2003. Disruption mutations of ADA2b and GCN5 transcriptional adapter genes dramatically affect Arabidopsis growth, development, and gene expression. Plant Cell

Wood CC, Roberson M, Tanner G, Peacock WJ, Dennis ES, Helliwell CA 2006. The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proc Natl Acdad Sci

Wang JW 2014. Regulation of flowering time by the miR156-mediated age pathway. Journal of Experimental Botany.

Wang HB, Wang LJ, Erdjument-Bromage H 2004. Role of histone H2A ubiquitination in polycomb silencing. Nature

Whetstine JR, Nottke A, Lan F, Huarte M, Smolikov S, Chen Z, Sooner E, Li E, Zhang G, Colaiacovo M, Shi Y 2006. Reversal of histone lysine trimethylation by the JMJD family of histone demethylases. Cell

Wu K, Malik K, Tian L, Brown D, Miki B 2000. Functional analysis of a RPD3 histone deacetylase homologue in *Arabidopsis thaliana*. Plant Mol. Biol.

Wu K, Tian L, Malik K, Brown D, Miki B (2000) Functional analysis of HD2 histone deacetylase homologues in Arabidopsis thaliana

Yang H, Howard M, Dean C. 2014. Antagonistic role for H3K36me3 and H3K27me3 in the cold-induced epigenetic switch at Arabidopsis FLC. Current Biology

Yang W, Jiang D, Jiang J, He Y 2010. A plant-specific histone H3 lysine 4 demethylase represses the floral transition in *Arabidopsis*. Plant J

Yang XJ, Seto EY 2007. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene

Yu X, Michaels SD 2010. The Arabidopsis Paf1c complex component CDC73 participates in the modification of FLOWERING LOCUS C chromatin. Plant Physiology

Yu CW, Liu X, Luo M, Chen C, Lin X, Tian G 2011. HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in *Arabidopsis*. Plant Physiol

Zhang H, Van Nocker S 2002. The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of FLOWERING LOCUS C. Plant Journal

Zhang H, Ransom C, Ludwig P, Van Nocker S 2003. Genetic analysis of early flowering mutants in Arabidopsis defines a class of pleotropic developmental regulator required for expression of the flowering-time switch flowering locus C. Genetics.

Zhao Z, Yu Y, Meyer D, Wu C, Shen WH 2005. Prevention of early flowering by expression of FLOWERING LOCUS C requires methylation of histone H3 K36. Nat.Cell Biol

Zhou C, Zhang L, Duan J, Miki B, Wu K 2005. HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. Plant Cell

Zhou C, Labbe H, Sridha S, Wang L, Tian L, Latoszek-Green M, Yang Z, Brown D, Miki B, Wu K 2004. Expression and function of HD2-type histone deacetylases in Arabidopsis development. Plant J

Zhu B, Zheng Y, Pham AD, Mandal SS, Erdjument-Bromage H, Tempst P, Reinberg D. Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. Mol. Cell